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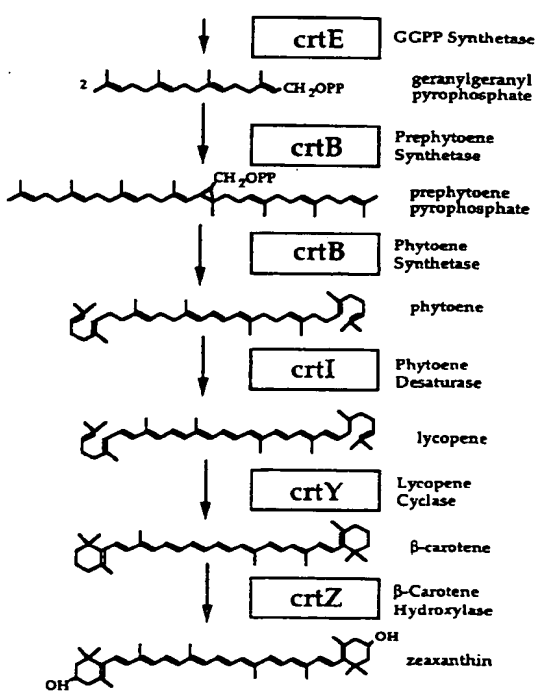
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(54) Fermentative carotenoid production

(57) The present invention is directed to a DNA sequence comprising one or more DNA sequences selected from the group consisting of a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE), a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB), a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI), a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which encodes the β -carotene hydroxylase of *Flavobacterium* sp. R1534 (crtZ) or DNA sequences which are substantially homologous, vectors comprising such DNA sequences and/or a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous, cells which are transformed by such DNA sequences and/or vectors, a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such transformed cells and a process for the preparation of a food or feed composition.

Fig. 1



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Description

Over 600 different carotenoids have been described from carotenogenic organisms found among bacteria, yeast, fungi and plants. Currently only two of them, β -carotene and astaxanthin are commercially produced in microorganisms and used in the food and feed industry. β -carotene is obtained from algae and astaxanthin is produced in *Pfaffia* strains which have been generated by classical mutation. However, fermentation in *Pfaffia* has the disadvantage of long fermentation cycles and recovery from algae is cumbersome. Therefore it is desirable to develop production systems which have better industrial applicability, e.g. can be manipulated for increased titers and/or reduced fermentation times. Two such systems using the biosynthetic genes from *Erwinia herbicola* and *Erwinia uredovora* have already been described in WO 91/13078 and EP 393 690, respectively. Furthermore, three β -carotene ketolase genes (β -carotene β -4-oxygenase) of the marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes* strain PC-1 (crtW) [Misawa, 1995, Biochem. Biophys. Res. Com. 209, 867-876][Misawa, 1995, J. Bacteriology 177, 6575-6584] and from the green algae *Haematococcus pluvialis* (bkt) [Lotan, 1995, FEBS Letters 364, 125-128][Kajiwara, 1995, Plant Mol. Biol. 29, 343-352] have been cloned. *E. coli* carrying either the carotenogenic genes (crtE, crtB, crtY and crtI) of *E. herbicola* [Hundle, 1994, MGG 245, 406-416] or of *E. uredovora* and complemented with the crtW gene of *A. aurantiacum* [Misawa, 1995] or the bkt gene of *H. pluvialis* [Lotan, 1995][Kajiwara, 1995] resulted in the accumulation of canthaxanthin (β , β -carotene-4,4'-dione), originating from the conversion of β -carotene, via the intermediate echinenone (β , β -carotene-4-one). Introduction of the above mentioned genes (crtW or bkt) into *E. coli* cells harbouring besides the carotenoid biosynthesis genes mentioned above also the crtZ gene of *E. uredovora* [Kajiwara, 1995][Misawa, 1995], resulted in both cases in the accumulation of astaxanthin (3,3'-dihydroxy- β , β -carotene-4,4'-dione). The results obtained with the bkt gene, are in contrast to the observation made by others [Lotan, 1995], who using the same experimental set-up, but introducing the *H. pluvialis* bkt gene in a zeaxanthin (β , β -carotene-3,3'-diol) synthesising *E. coli* host harbouring the carotenoid biosynthesis genes of *E. herbicola*, a close relative of the above mentioned *E. uredovora* strain, did not observe astaxanthin production.

However, functionally active combinations of the carotenoid biosynthesising genes of the present invention with the known crtW genes have not been shown so far and even more importantly there is a continuing need in even more optimized fermentation systems for industrial application.

It is therefore an object of the present invention to provide a DNA sequence comprising one or more DNA sequences selected from the group consisting of:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous;

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous;

e) a DNA sequence which encodes the β -carotene hydroxylase of *Flavobacterium* sp. R1534 (crtZ) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable culture conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid or carotenoid mixture is added to food or feed.

Furthermore, a DNA sequence comprising the following DNA sequences is an object of the present invention:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous, and

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous, and

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R 1534 (crtI) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of lycopene and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably lycopene or carotenoid mixture, preferably a lycopene comprising mixture is added to food or feed.

Furthermore a DNA sequence comprising the following DNA sequence is also an object of the present invention:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous, and

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous, and

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous, and

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of β -carotene and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably β -carotene or carotenoid mixture, preferably a β -carotene comprising mixture is added to food or feed.

Furthermore a cell which is transformed by the above mentioned DNA sequence comprising subsequences a) to d) or the vector comprising it and a second DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous or a second vector which comprises a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous; and a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of echinenone and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably echinenone or carotenoid mixture, preferably an echinenone comprising mixture is added to food or feed.

Furthermore it is an object of the present invention to provide a DNA sequence as mentioned above comprising subsequences a) to d) and a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous and a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an

object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable culture conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells of the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, especially such a process for the preparation of echinenone or canthaxanthin and a process for the preparation of a food or feed compositing characterized therein that after such a process has been effected the carotenoid, preferably echinenone or canthaxanthin or carotenoid mixture, preferably a echinenone or canthaxanthin containing mixture is added to food or feed.

Furthermore a DNA sequence comprising the following DNA sequences is also an object of the present invention:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous, and

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous, and

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous, and

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous, and

e) a DNA sequence which encodes the β -carotene hydroxylase of *Flavobacterium* sp. R1534 (crtZ) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells of the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of zeaxanthin and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably zeaxanthin or the carotenoid mixture, preferably a zeaxanthin containing mixture is added to food or feed.

Furthermore a DNA sequence as mentioned above comprising subsequences a) to e) and in addition a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous is an object of the present invention and to provide a vector comprising such DNA sequence, preferably in form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable culture conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells of the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of zeaxanthin, adonixanthin or astaxanthin and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably zeaxanthin, adonixanthin or astaxanthin or carotenoid mixture, preferably a zeaxanthin, adonixanthin or astaxanthin containing mixture is added to food or feed.

Furthermore a cell which is transformed by the DNA sequence mentioned above comprising subsequences a) to e) or a vector comprising such DNA sequence and a second DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous or a second vector which comprises a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous is also an object of the present invention and a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium, and in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of zeaxanthin or adonixanthin and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably zeaxanthin or

adonixanthin or carotenoid mixture, preferably a zeaxanthin or adonixanthin containing mixture is added to food or feed.

In this context it should be mentioned that the expression "a DNA sequence is substantially homologous" refers with respect to the crtE encoding DNA sequence to a DNA sequence which encodes an amino acid sequence which shows more than 45 %, preferably more than 60 % and more preferably more than 75 % and most preferably more than 90 % identical amino acids when compared to the amino acid sequence of crtE of *Flavobacterium* sp. 1534 and is the amino acid sequence of a polypeptide which shows the same type of enzymatic activity as the enzyme encoded by crtE of *Flavobacterium* sp. 1534. In analogy with respect to crtB this means more than 60 %, preferably more than 70 %, more preferably more than 80 % and most preferably more than 90 %; with respect to crtI this means more than 70 %, preferably more than 80 % and most preferably more than 90 %; with respect to crtY this means 55 %, preferably 70 %, more preferably 80 % and most preferably 90 %; with respect to crtZ this means more than 60 %, preferably 70 %, more preferably 80 % and most preferably 90 %; with respect to crt W this also means more than 60 %, preferably 70 %, more preferably 80 % and most preferably 90 %. Sequences which are substantially homologous to crt W are known, e.g. in form of the β -carotene β 4-oxygenase of *Agrobacterium aurantiacum* or the green algae *Haematococcus pluvialis* (bkt).

DNA sequences in form of genomic DNA, cDNA or synthetic DNA can be prepared as known in the art [see e.g. Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press 1989] or, e.g. as specifically described in Examples 1, 2 or 7.

The cloning of the DNA-sequences of the present invention from such genomic DNA can than be effected, e.g. by using the well known polymerase chain reaction (PCR) method. The principles of this method are outlined e.g. in *PCR Protocols: A guide to Methods and Applications*, Academic Press, Inc. (1990). PCR is an in vitro method for producing large amounts of a specific DNA of defined length and sequence from a mixture of different DNA-sequences. Thereby, PCR is based on the enzymatic amplification of the specific DNA fragment of interest which is flanked by two oligonucleotide primers which are specific for this sequence and which hybridize to the opposite strand of the target sequence. The primers are oriented with their 3' ends pointing toward each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment between the PCR primers. Since the extension product of each primer can serve as a template for the other, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. By utilizing the thermostable Taq DNA polymerase, isolated from the thermophilic bacteria *Thermus aquaticus*, it has been possible to avoid denaturation of the polymerase which necessitated the addition of enzyme after each heat denaturation step. This development has led to the automation of PCR by a variety of simple temperature-cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. The increased specificity improves the overall yield of amplified products by minimizing the competition by non-target fragments for enzyme and primers. In this way the specific sequence of interest is highly amplified and can be easily separated from the non-specific sequences by methods known in the art, e.g. by separation on an agarose gel and cloned by methods known in the art using vectors as described e.g. by Holten and Graham in *Nucleic Acid Res.* **19**, 1156 (1991), Kovalic et al. in *Nucleic Acid Res.* **19**, 4560 (1991), Marchuk et al. in *Nucleic Acid Res.* **19**, 1154 (1991) or Mead et al. in *Bio/Technology* **9**, 657-663 (1991).

The oligonucleotide primers used in the PCR procedure can be prepared as known in the art and described e.g. in Sambrook et al., s.a.

Amplified DNA-sequences can than be used to screen DNA libraries by methods known in the art (Sambrook et al., s.a.) or as specifically described in Examples 1 and 2.

Once complete DNA-sequences of the present invention have been obtained they can be used as a guideline to define new PCR primers for the cloning of substantially homologous DNA sequences from other sources. In addition they and such homologous DNA sequences can be integrated into vectors by methods known in the art and described e.g. in Sambrook et al. (s.a.) to express or overexpress the encoded polypeptide(s) in appropriate host systems. However, a man skilled in the art knows that also the DNA-sequences themselves can be used to transform the suitable host systems of the invention to get overexpression of the encoded polypeptide. Appropriate host systems are for example Bacteria e.g. *E. coli*, Bacilli as, e.g. *Bacillus subtilis* or *Flavobacter* strains. *E. coli*, which could be used are *E. coli* K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in *J. Bacteriol.* **120**, 466-474 (1974)], HB 101 [ATCC No. 33694] or *E. coli* SG13009 [Gottesman et al., *J. Bacteriol.* **148**, 265-273 (1981)]. Suitable eukaryotic host systems are for example fungi, like *Aspergilli*, e.g. *Aspergillus niger* [ATCC 9142] or yeasts, like *Saccharomyces*, e.g. *Saccharomyces cerevisiae* or *Pichia*, like *pastoris*, all available from ATCC.

Suitable vectors which can be used for expression in *E. coli* are mentioned, e.g. by Sambrook et al. [s.a.] or by Fiers et al. in *Proc. 8th Int. Biotechnology Symposium* [Soc. Franc. de Microbiol., Paris (Durand et al., eds.), pp. 680-697 (1988)] or by Bujard et al. in *Methods in Enzymology*, eds. Wu and Grossmann, Academic Press, Inc. Vol. **155**, 416-433 (1987) and Stüber et al. in *Immunological Methods*, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. **IV**, 121-152 (1990). Vectors which could be used for expression in Bacilli are known in the art and described, e.g. in EP 405 370, EP 635 572 *Proc. Nat. Acad. Sci. USA* **81**, 439 (1984) by Yansura and Henner, *Meth. Enzym.* **185**, 199-228 (1990) or EP 207 459. Vectors which can be used for expression in fungi are known in the art and described e.g. in EP 420 358 and for yeast in EP 183 070, EP 183 071, EP 248 227, EP 263 311.

Once such DNA-sequences have been expressed in an appropriate host cell in a suitable medium the carotenoids can be isolated either from the medium in the case they are secreted into the medium or from the host organism and, if necessary separated from other carotenoids if present in case one specific carotenoid is desired by methods known in the art (see e.g. Carotenoids Vol IA: Isolation and Analysis, G. Britton, S. Liaaen-Jensen, H. Pfander; 1995, Birkhäuser Verlag, Basel).

The carotenoids of the present invention can be used in a process for the preparation of food or feeds. A man skilled in the art is familiar with such process. Such compound foods or feeds can further comprise additives or components generally used for such purpose and known in the state of the art.

After the invention has been described in general hereinbefore, the following figures and examples are intended to illustrate details of the invention, without thereby limiting it in any matter.

Figure 1: The biosynthesis pathway for the formation of carotenoids of *Flavobacterium* sp. R1534 is illustrated explaining the enzymatic activities which are encoded by DNA sequences of the present invention.

Figure 2: Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with the restriction enzymes shown on top of each lane and hybridized with Probe 46F. The arrow indicated the isolated 2.4 kb XhoI/PstI fragment.

Figure 3: Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with ClaI or double digested with ClaI and HindIII. Blots shown in Panel A and B were hybridized to probe A or probe B, respectively (see examples). Both ClaI/HindIII fragments of 1.8 kb and 9.2 kb are indicated.

Figure 4: Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with the restriction enzymes shown on top of each lane and hybridized to probe C. The isolated 2.8 kb SaI/HindIII fragment is shown by the arrow.

Figure 5: Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with the restriction enzymes shown on top of each lane and hybridized to probe D. The isolated BclI/SphI fragment of approx. 3 kb is shown by the arrow.

Figure 6: Physical map of the organization of the carotenoid biosynthesis cluster in *Flavobacterium* sp. R1534, deduced from the genomic clones obtained. The location of the probes used for the screening are shown as bars on the respective clones.

Figure 7: Nucleotide sequence of the *Flavobacterium* sp. R1534 carotenoid biosynthesis cluster and its flanking regions. The nucleotide sequence is numbered from the first nucleotide shown (see BamHI site of Fig. 6). The deduced amino acid sequence of the ORF's (orf-5, orf-1, crtE, crtB, crtI, crtY, crtZ and orf-16) are shown with the single-letter amino acid code. Arrow (→) indicate the direction of the transcription; asterisks, stop codons.

Figure 8: Protein sequence of the GGPP synthase (crtE) of *Flavobacterium* sp. R1534 with a MW of 31331 Da.

Figure 9: Protein sequence of the prephytoene synthetase (crtB) of *Flavobacterium* sp. R1534 with a MW of 32615 Da.

Figure 10: Protein sequence of the phytoene desaturase (crtI) of *Flavobacterium* sp. R1534 with a MW of 54411 Da.

Figure 11: Protein sequence of the lycopene cyclase (crtY) of *Flavobacterium* sp. R1534 with a MW of 42368 Da.

Figure 12: Protein sequence of the β -carotene hydroxylase (crtZ) of *Flavobacterium* sp. R1534 with a MW of 19282 Da.

Figure 13: Recombinant plasmids containing deletions of the *Flavobacterium* sp. R1534 carotenoid biosynthesis gene cluster.

Figure 14: Primers used for PCR reactions. The underlined sequence is the recognition site of the indicated restriction enzyme. Small caps indicate nucleotides introduced by mutagenesis. Boxes show the artificial RBS which is recognized in *B. subtilis*. Small caps in bold show the location of the original adenine creating

the translation start site (ATG) of the following gene (see original operon). All the ATG's of the original *Flavobacter* carotenoid biosynthetic genes had to be destroyed to not interfere with the rebuild transcription start site. Arrows indicate start and ends of the indicated *Flavobacterium* R1534 WT carotenoid genes.

Figure 15: Linkers used for the different constructions. The underlined sequence is the recognition site of the indicated restriction enzyme. Small caps indicate nucleotides introduced by synthetic primers. Boxes show the artificial RBS which is recognized in *B. subtilis*. Arrows indicate start and ends of the indicated *Flavobacterium* carotenoid genes.

Figure 16: Construction of plasmids pBIKS(+)-clone59-2, pLyco and pZea4.

Figure 17: Construction of plasmid p602CAR.

Figure 18: Construction of plasmids pBIKS(+)-CARVEG-E and p602 CARVEG-E.

Figure 19: Construction of plasmids pHP13-2CARZYIB-EINV and pHP13-2PN25ZYIB-EINV.

Figure 20: Construction of plasmid pXI12-ZYIB-EINVMUTRBS2C.

Figure 21: Northern blot analysis of *B. subtilis* strain BS1012::ZYIB-EINV4. Panel A: Schematic representation of a reciprocal integration of plasmid pXI12-ZYIB-EINV4 into the levan-sucrase gene of *B. subtilis*. Panel B: Northern blot obtained with probe A (PCR fragment which was obtained with CAR 51 and CAR 76 and hybridizes to the 3' end of *crtZ* and the 5' end of *crtY*). Panel C: Northern blot obtained with probe B (BamHI-XhoI fragment isolated from plasmid pBIKS(+)-*crtE/2* and hybridizing to the 5' part of the *crtE* gene).

Figure 22: Schematic representation of the integration sites of three transformed *Bacillus subtilis* strains: BS1012::SFCO, BS1012::SFCOCAT1 and BA1012::SFCONEO1. Amplification of the synthetic *Flavobacterium* carotenoid operon (SFCO) can only be obtained in those strains having amplifiable structures. Probe A was used to determine the copy number of the integrated SFCO. Erythromycin resistance gene (*ermAM*), chloramphenicol resistance gene (*cat*), neomycin resistance gene (*neo*), terminator of the *cryT* gene of *B. subtilis* (*cryT*), levan-sucrase gene (*sac-B* 5' and *sac-B* 3'), plasmid sequences of pXI12 (pXI12), promoter originating from site I of the *veg* promoter complex (PvegI).

Figure 23: Construction of plasmids pXI12-ZYIB-EINV4MUTRBS2CNEO and pXI12-ZYIB-EINV4MUTRBS2CCAT.

Figure 24: Complete nucleotide sequence of plasmid pZea4.

Figure 25: Synthetic *crtW* gene of *Alcaligenes* PC-1. The translated protein sequence is shown above the double stranded DNA sequence. The twelve oligonucleotides (*crtW*1-*crtW*12) used for the PCR synthesis are underlined.

Figure 26: Construction of plasmid pBIKS-*crtEBIYZW*. The HindIII-Pm1I fragment of pALTER-Ex2-*crtW*, carrying the synthetic *crtW* gene, was cloned into the HindIII and MluI (blunt) sites. PvegI and Ptac are the promoters used for the transcription of the two operons. The ColE1 replication origin of this plasmid is compatible with the p15A origin present in the pALTER-Ex2 constructs.

Figure 27: Relevant inserts of all plasmids constructed in Example 7. Disrupted genes are shown by //. Restriction sites: S=SacI, X=XbaI, H=HindIII, N=NsiI, Hp=HpaI, Nd=NdeI.

Figure 28: Reaction products (carotenoids) obtained from β -carotene by the process of the present invention.

Example 1

Materials and general methods used

Bacterial strains and plasmids: *Flavobacterium* sp. R1534 WT (ATCC 21588) was the DNA source for the genes

cloned. Partial genomic libraries of *Flavobacterium* sp. R1534 WT DNA were constructed into the pBluescriptII+(KS) or (SK) vector (Stratagene, La Jolla, USA) and transformed into *E. coli* XL-1 blue (Stratagene) or JM109.

Media and growth conditions: Transformed *E. coli* were grown in Luria broth (LB) at 37°C with 100 µg Ampicillin (Amp)/ml for selection. *Flavobacterium* sp. R1534 WT was grown at 27°C in medium containing 1% glucose, 1% tryptone (Difco Laboratories), 1% yeast extract (Difco), 0.5% MgSO₄ 7H₂O and 3% NaCl.

Colony screening: Screening of the *E. coli* transformants was done by PCR basically according to the method described by Zon et al. [Zon et al., *BioTechniques* **7**, 696-698 (1989)] using the following primers:

Primer #7: 5'-CCTGGATGACGTGCTGGAATATTCC-3'

Primer #8: 5'-CAAGGCCCAGATCGCAGGCG-3'

Genomic DNA: A 50 ml overnight culture of *Flavobacterium* sp. R1534 was centrifuged at 10,000 g for 10 minutes. The pellet was washed briefly with 10 ml of lysis buffer (50 mM EDTA, 0.1M NaCl pH7.5), resuspended in 4 ml of the same buffer supplemented with 10 mg of lysozyme and incubated at 37°C for 15 minutes. After addition of 0.3 ml of N-Lauroyl sarcosine (20%) the incubation at 37°C was continued for another 15 minutes before the extraction of the DNA with phenol, phenol/chloroform and chloroform. The DNA was ethanol precipitated at room temperature for 20 minutes in the presence of 0.3 M sodium acetate (pH 5.2), followed by centrifugation at 10,000 g for 15 minutes. The pellet was rinsed with 70% ethanol, dried and resuspended in 1 ml of TE (10 mM Tris, 1mM EDTA, pH 8.0).

All genomic DNA used in the southern blot analysis and cloning experiments was dialysed against H₂O for 48 hours, using collodium bags (Sartorius, Germany), ethanol precipitated in the presence of 0.3 M sodium acetate and resuspended in H₂O.

Probe labelling: DNA probes were labeled with (α -³²P) dGTP (Amersham) by random-priming according to [Sambrook et al., s.a.].

Probes used to screen the mini-libraries: Probe 46F is a 119 bp fragment obtained by PCR using primer #7 and #8 and *Flavobacterium* sp. R1534 genomic DNA as template. This probe was proposed to be a fragment of the *Flavobacterium* sp. R1534 phytoene synthase (crtB) gene, since it shows significant homology to the phytoene synthase genes from other species (e.g. *E. uredovora*, *E. herbicola*). **Probe A** is a BstXI - PstI fragment of 184 bp originating from the right arm of the insert of clone 85. **Probe B** is a 397 bp XhoI - NotI fragment obtained from the left end of the insert of clone 85. **Probe C** is a 536 bp BglII - PstI fragment from the right end of the insert of clone 85. **Probe D** is a 376 bp KpnI - BstYI fragment isolated from the insert of clone 59. The localization of the individual probes is shown in figure 6.

Oligonucleotide synthesis: The oligonucleotides used for PCR reactions or for sequencing were synthesized with an Applied Biosystems 392 DNA synthesizer.

Southern blot analysis: For hybridization experiments *Flavobacterium* sp. R1534 genomic DNA (3 µg) was digested with the appropriate restriction enzymes and electrophoresed on a 0.75% agarose gel. The transfer to Zeta-Probe blotting membranes (BIO-RAD), was done as described [Southern, E.M., *J. Mol. Biol.* **98**, 503 (1975)]. Prehybridization and hybridization was in 7%SDS, 1% BSA (fraction V; Boehringer), 0.5M Na₂HPO₄, pH 7.2 at 65°C. After hybridization the membranes were washed twice for 5 minutes in 2x SSC, 1% SDS at room temperature and twice for 15 minutes in 0.1% SSC, 0.1% SDS at 65°C.

DNA sequence analysis: The sequence was determined by the dideoxy chain termination technique [Sanger et al., *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467 (1977)] using the Sequenase Kit (United States Biochemical). Both strands were completely sequenced and the sequence analyzed using the GCG sequence analysis software package (Version 8.0) by Genetics Computer, Inc. [Devereux et al., *Nucleic Acids. Res.* **12**, 387-395 (1984)].

Analysis of carotenoids: *E. coli* XL-1 or JM109 cells (200 - 400 ml) carrying different plasmid constructs were grown for the times indicated in the text, usually 24 to 60 hours, in LB supplemented with 100 µg Ampicillin/ml, in shake flasks at 37°C and 220 rpm.

The carotenoids present in the microorganisms were extracted with an adequate volume of acetone using a rotation homogenizer (Polytron, Kinematica AG, CH-Luzern). The homogenate was filtered through the sintered glass of a suction filter into a round bottom flask. The filtrate was evaporated by means of a rotation evaporator at 50°C using a water-jet vacuum. For the zeaxanthin detection the residue was dissolved in n-hexane/acetone (86:14) before analysis with a normalphase HPLC as described in [Weber, S. in *Analytical Methods for Vitamins and Carotenoids in Feed*, Keller, H.E., Editor, 83-85 (1988)]. For the detection of β -carotene and lycopene the evaporated extract was dissolved in n-hexane/acetone (99:1) and analysed by HPLC as described in [Hengartner et al., *Helv. Chim. Acta* **75**, 1848-1865 (1992)].

Example 2**Cloning of the *Flavobacterium* sp. R1534 carotenoid biosynthetic genes.**

To identify and isolate DNA fragments carrying the genes of the carotenoid biosynthesis pathway, we used the DNA fragment 46F (see methods) to probe a Southern Blot carrying chromosomal DNA of *Flavobacterium* sp. R1534 digested with different restriction enzymes Fig. 2. The 2.4 kb XhoI/PstI fragment hybridizing to the probe seemed the most appropriate one to start with. Genomic *Flavobacterium* sp. R1534 DNA was digested with XhoI/PstI and run on a 1% agarose gel. According to a comigrating DNA marker, the region of about 2.4 kb was cut out of the gel and the DNA isolated. A XhoI/PstI mini library of *Flavobacterium* sp. R1534 genomic DNA was constructed into XhoI - PstI sites of pBluescriptIIKS(+). One hundred *E. coli* XL1 transformants were subsequently screened by PCR with primer #7 and primer #8, the same primers previously used to obtain the 119 bp fragment (46F). One positive transformant, named done 85, was found. Sequencing of the insert revealed sequences not only homologous to the phytoene synthase (crtB) but also to the phytoene desaturase (crtI) of both *Erwinia* species *herbicola* and *uredovora*. Left and right hand genomic sequences of done 85 were obtained by the same approach using probe A and probe B. *Flavobacterium* sp. R1534 genomic DNA was double digested with ClaI and Hind III and subjected to Southern analysis with probe A and probe B. With probe A a ClaI/HindIII fragment of approx. 1.8 kb was identified (Fig. 3A), isolated and subcloned into the ClaI/HindIII sites of pBluescriptIIKS (+). Screening of the *E. coli* XL1 transformants with probe A gave 6 positive clones. The insert of one of these positives, clone 43-3, was sequenced and showed homology to the N-terminus of crtI genes and to the C-terminus of crtY genes of both *Erwinia* species mentioned above. With probe B an approx. 9.2 kb ClaI/HindIII fragment was detected (Fig. 3B), isolated and subcloned into pBluescriptIIKS (+).

A screening of the transformants gave one positive, clone 51. Sequencing of the 5' and 3' of the insert, revealed that only the region close to the HindIII site showed relevant homology to genes of the carotenoid biosynthesis of the *Erwinia* species mentioned above (e.g. crtB gene and crtE gene). The sequence around the ClaI site showed no homology to known genes of the carotenoid biosynthesis pathway. Based on this information and to facilitate further sequencing and construction work, the 4.2 kb BamHI/HindIII fragment of done 51 was subcloned into the respective sites of pBluescriptIIKS(+) resulting in clone 2. Sequencing of the insert of this clone confirmed the presence of genes homologous to *Erwinia* sp. crtB and crtE genes. These genes were located within 1.8 kb from the HindIII site. The remaining 2.4 kb of this insert had no homology to known carotenoid biosynthesis genes.

Additional genomic sequences downstream of the ClaI site were detected using probe C to hybridize to *Flavobacterium* sp. R1534 genomic DNA digested with different restriction enzymes (see figure 4).

A SalI/HindIII fragment of 2.8 kb identified by Southern analysis was isolated and subcloned into the HindIII/XhoI sites of pBluescriptIIKS (+). Screening of the *E. coli* XL1 transformants with probe A gave one positive clone named clone 59. The insert of this clone confirmed the sequence of clone 43-3 and contained in addition sequences homologous to the N-terminus of the crtY gene from other known lycopene cyclases. To obtain the putative missing crtZ gene a Sau3AI partial digestion library of *Flavobacterium* sp. R1534 was constructed into the BamHI site of pBluescriptIIKS(+). Screening of this library with probe D gave several positive clones. One transformant designated, clone 6a, had an insert of 4.9 kb. Sequencing of the insert revealed besides the already known sequences coding for crtB, crtI and crtY also the missing crtZ gene. Clone 7g was isolated from a mini library carrying BclI/SphI fragments of R1534 (Fig. 5) and screened with probe D. The insert size of done 7g is approx. 3 kb.

The six independent inserts of the clones described above covering approx. 14 kb of the *Flavobacterium* sp. R1534 genome are compiled in Figure 6.

The determined sequence spanning from the BamHI site (position 1) to base pair 8625 is shown figure 7.

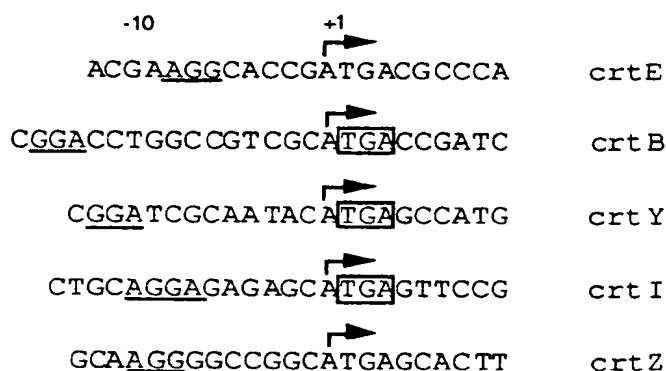
Putative protein coding regions of the cloned R1534 sequence.

Computer analysis using the CodonPreference program of the GCG package, which recognizes protein coding regions by virtue of the similarity of their codon usage to a given codon frequency table, revealed eight open reading frames (ORFs) encoding putative proteins: a partial ORF from 1 to 1165 (ORF-5) coding for a polypeptide larger than 41382 Da; an ORF coding for a polypeptide with a molecular weight of 40081 Da from 1180 to 2352 (ORF-1); an ORF coding for a polypeptide with a molecular weight of 31331 Da from 2521 to 3405 (crtE); an ORF coding for a polypeptide with a molecular weight of 32615 Da from 4316 to 3408 (crtB); an ORF coding for a polypeptide with a molecular weight of 54411 Da from 5797 to 4316 (crtI); an ORF coding for a polypeptide with a molecular weight of 42368 Da from 6942 to 5797 (crtY); an ORF coding for a polypeptide with a molecular weight of 19282 Da from 7448 to 6942 (crtZ); and an ORF coding for a polypeptide with a molecular weight of 19368 Da from 8315 to 7770 (ORF-16); ORF-1 and crtE have the opposite transcriptional orientation from the others (Fig. 6). The translation start sites of the ORFs crtI, crtY and crtZ could clearly be determined based on the appropriately located sequences homologous to the Shine/Dalgarno (S/D) [Shine and Dalgarno, Proc. Natl. Acad. Sci. USA 71, 1342-1346 (1974)] consensus sequence AGG-6-9N-ATG (Fig. 10) and the homology to the N-terminal sequences of the respective enzymes of *E. herbicola* and *E. uredovora*. The

translation of the ORF crtB could potentially start from three closely spaced codons ATG (4316), ATG (4241) and ATG (4211). The first one, although not having the best S/D sequence of the three, gives a translation product with the highest homology to the N-terminus of the *E. herbicola* and *E. uredovora* crtB protein, and is therefore the most likely translation start site. The translation of ORF crtE could potentially start from five different start codons found within 150 bp :
 5 ATG (2389), ATG (2446), ATG (2473), ATG (2497) and ATG (2521). We believe that based on the following observations, the ATG (2521) is the most likely transcription start site of crtE: this ATG start codon is preceded by the best consensus S/D sequence of all five putative start sites mentioned; and the putative N-terminal amino acid sequence of the protein encoded has the highest homology to the N-terminus of the crtE enzymes of *E. herbicola* and *E. uredovora*;

10 Characteristics of the crt translational initiation sites and gene products.

The translational start sites of the five carotenoid biosynthesis genes are shown below and the possible ribosome binding sites are underlined. The genes crtZ, crtY, crtI and crtB are grouped so tightly that the TGA stop codon of the anterior gene overlaps the ATG of the following gene. Only three of the five genes (crtI, crtY and crtZ) fit with the consensus for optimal S/D sequences. The boxed TGA sequence shows the stop codon of the anterior gene.
 15



35 Amino acid sequences of individual crt genes of *Flavobacterium* sp. R1534.

All five ORFs of *Flavobacterium* sp. R1534 having homology to known carotenoid biosynthesis genes of other species are clustered in approx. 5.2 kb of the sequence (Fig. 7).
 40

GGDP synthase (crtE)

The amino acid (aa) sequence of the geranylgeranyl pyrophosphate synthase (crtE gene product) consists of 295 aa and is shown in figure 8. This enzyme condenses farnesyl pyrophosphate and isopentenyl pyrophosphate in a 1' - 4.
 45

Phytoene synthase (crtB)

This enzyme catalyzes two enzymatic steps. First it condenses in a head to head reaction two geranylgeranyl pyrophosphates (C20) to the C40 carotenoid prephytoene. Second it rearranges the cyclopropylring of prephytoene to phytoene.
 50 The 303 aa encoded by the crtB gene of *Flavobacterium* sp. R1534 is shown in figure 9.

Phytoene desaturase (crtI)

The phytoene desaturase of *Flavobacterium* sp. R1534 consisting of 494 aa, shown in figure 10, performs like the
 55 crtI enzyme of *E. herbicola* and *E. uredovora*, four desaturation steps, converting the non-coloured carotenoid phytoene to the red coloured lycopene.

Lycopene cyclase (crtY)

The crtY gene product of *Flavobacterium* sp. R1534 is sufficient to introduce the β -ionone rings at both sides of lycopene to obtain β -carotene. The lycopene cyclase of *Flavobacterium* sp. R1534 consists of 382 aa (Fig. 11).

 β -carotene hydroxylase (crtZ)

The gene product of crtZ consisting of 169 aa (Fig. 12) and hydroxylates β -carotene to the xanthophyll zeaxanthin.

Putative enzymatic functions of the ORF's (orf-1, orf-5 and orf-16)

The orf-1 has at the aa level over 40% identity to acetoacetyl-CoA thiolases of different organisms (e.g. *Candida tropicalis*, human, rat). This gene is therefore most likely a putative acetoacetyl-CoA thiolase (acetyl-CoA acetyltransferase), which condenses two molecules of acetyl-CoA to Acetoacetyl-CoA. Condensation of acetoacetyl-CoA with a third acetyl-CoA by the HMG-CoA synthase forms β -hydroxy- β -methylglutaryl-CoA (HMG-CoA). This compound is part of the mevalonate pathway which produces besides sterols also numerous kinds of isoprenoids with diverse cellular functions. In bacteria and plants, the isoprenoid pathway is also able to synthesize some unique products like carotenoids, growth regulators (e.g. in plants gibberellins and abscisic acid) and secondary metabolites like phytoalexins [Riou et al., Gene 148, 293-297 (1994)].

The orf-5 has a low homology of approx. 30%, to the amino acid sequence of polyketide synthases from different streptomycetes (e.g. *S. violaceoruber*, *S. cinnamomensis*). These antibiotic synthesizing enzymes (polyketide synthases), have been classified into two groups. Type-I polyketide synthases are large multifunctional proteins, whereas type-II polyketide synthases are multiprotein complexes composed of several individual proteins involved in the subreactions of the polyketide synthesis [Bibb, et al. Gene 142, 31-39 (1994)].

The putative protein encoded by the orf-16 has at the aa level an identity of 42% when compared to the soluble hydrogenase subunit of *Anabaena cylindrica*.

Functional assignment of the ORF's (crtE, crtB, crtI, crtY and crtZ) to enzymatic activities of the carotenoid biosynthesis pathway.

The biochemical assignment of the gene products of the different ORF's were revealed by analyzing carotenoid accumulation in *E. coli* host strains that were transformed with deleted variants of the *Flavobacterium* sp. gene cluster and thus expressed not all of the crt genes (Fig. 13).

Three different plasmids were constructed: pLyco, p59-2 and pZea4. Plasmid p59-2 was obtained by subcloning the HindIII/BamHI fragment of clone 2 into the HindIII/BamHI sites of clone 59. p59-2 carries the ORF's of the crtE, crtB, crtI and crtY gene and should lead to the production of β -carotene. pLyco was obtained by deleting the KpnI/KpnI fragment, coding for approx. one half (N-terminus) of the crtY gene, from the p59-2 plasmid. *E. coli* cells transformed with pLyco, and therefore having a truncated non-functional crtY gene, should produce lycopene, the precursor of β -carotene. pZea4 was constructed by ligation of the AscI-SpeI fragment of p59-2, containing the crtE, crtB, crtI and most of the crtY gene with the AscI/XbaI fragment of clone 6a, containing the sequences to complete the crtY gene and the crtZ gene. pZea4 [for complete sequence see Fig. 24; nucleotides 1 to 683 result from pBluescriptIIKS(+), nucleotides 684 to 8961 from *Flavobacterium* R1534 WT genome, nucleotides 8962 to 11233 from pBluescriptIIKS(+)] has therefore all five ORF's of the zeaxanthin biosynthesis pathway. Plasmid pZea4 has been deposited on May 25, 1995 at the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany) under accession No. DSM 10012. *E. coli* cells transformed with this latter plasmid should therefore produce zeaxanthin. For the detection of the carotenoid produced, transformants were grown for 48 hours in shake flasks and then subjected to carotenoid analysis as described in the methods section. Figure 13 summarizes the different inserts of the plasmids described above, and the main carotenoid detected in the cells.

As expected the pLyco carrying *E. coli* cells produced lycopene, those carrying p59-2 produced β -carotene (all-E,9-Z,13-Z) and the cells having the pZea4 construct produced zeaxanthin. This confirms that all the necessary genes of *Flavobacterium* sp. R1534 for the synthesis of zeaxanthin or their precursors (phytoene, lycopene and β -carotene) were cloned.

Example 3**Materials and methods used for expression of carotenoid synthesizing enzymes**

Bacterial strains and plasmids: The vectors pBluescriptIIKS (+) or (-) (Stratagene, La Jolla, USA) and pUC18 [Vieira and Messing, Gene 19, 259-268 (1982); Norrander et al., Gene 26, 101-106 (1983)] were used for cloning in dif-

ferent *E. coli* strains, like XL-1 blue (Stratagene), TG1 or JM109. In all *B. subtilis* transformations, strain 1012 was used. Plasmids pHP13 [Haima et al., Mol. Gen. Genet. 209, 335-342 (1987)] and p602/22 [LeGrice, S.F.J. in Gene Expression Technology, Goeddel, D.V., Editor, 201-214 (1990)] are Gram (+)/(-) shuttle vectors able to replicate in *B. subtilis* and *E. coli* cells. Plasmid p205 contains the *vegI* promoter cloned into the *SmaI* site of pUC18. Plasmid pXI12 is an integration vector for the constitutive expression of genes in *B. subtilis* [Haiker et al., in 7th Int. Symposium on the Genetics of Industrial Microorganisms, June 26-July 1, 1994, Mongreal, Quebec, Canada (1994)]. Plasmid pBEST501 [Itaya et al., Nucleic Acids Res. 17 (11), 4410 (1989)] contains the neomycin resistance gene cassette originating from the plasmid pUB110 (GenBank entry: M19465) of *S. aureus* [McKenzie et al., Plasmid 15, 93-103 (1986); McKenzie et al., Plasmid 17, 83-84 (1987)]. This neomycin gene has been shown to work as a selection marker when present in a single copy in the genome of *B. subtilis*. Plasmid pC194 (ATCC 37034) (GenBank entry: L08860) originates from *S. aureus* [Horinouchi and Weisblau, J. Bacteriol. 150, 815-825 (1982)] and contains the chloramphenicol acetyltransferase gene.

Media and growth conditions: *E. coli* were grown in Luria broth (LB) at 37°C with 100 µg Ampicillin (Amp)/ml for selection. *B. subtilis* cells were grown in VY-medium supplemented with either erythromycin (1 µg/ml), neomycin (5-180 µg/ml) or chloramphenicol (10-80 µg/ml).

Transformation: *E. coli* transformations were done by electroporation using the Gen-pulser device of BIO-RAD (Hercules, CA, USA) with the following parameters (200 Ω, 250 µFD, 2.5V). *B. subtilis* transformations were done basically according to the standard procedure method 2.8 described by [Cutting and Vander Horn in Molecular Biological Methods for Bacillus, Harwood, C.R and Cutting, S.M., Editor, John Wiley & Sons: Chichester, England. 61-74 (1990)].

Colony screening: Bacterial colony screening was done as described by [Zon et al., s.a.].

Oligonucleotide synthesis: The oligonucleotides used for PCR reactions or for sequencing were synthesized with an Applied Biosystems 392 DNA synthesizer.

PCR reactions: The PCR reactions were performed using either the **UITma DNA polymerase** (Perkin Elmer Cetus) or the **Pfu Vent polymerase** (New England Biolabs) according to the manufacturers instructions. A typical 50 µl PCR reaction contained: 100ng of template DNA, 10 pM of each of the primers, all four dNTP's (final conc. 300 µM), MgCl₂ (when UITma polymerase was used; final conc. 2 mM), 1x UITma reaction buffer or 1x Pfu buffer (supplied by the manufacturer). All components of the reaction with the exception of the DNA polymerase were incubated at 95°C for 2 min. followed by the cycles indicated in the respective section (see below). In all reactions a hot start was made, by adding the polymerase in the first round of the cycle during the 72°C elongation step. At the end of the PCR reaction an aliquot was analysed on 1% agarose gel, before extracting once with phenol/chloroform. The amplified fragment in the aqueous phase was precipitated with 1/10 of a 3M NaAcetate solution and two volumes of Ethanol. After centrifugation for 5 min. at 12000 rpm, the pellet was resuspended in an adequate volume of H₂O, typically 40 µl, before digestion with the indicated restriction enzymes was performed. After the digestion the mixture was separated on a 1% low melting point agarose. The PCR product of the expected size were excised from the agarose and purified using the glass beads method (GENECLEAN KIT, Bio 101, Vista CA, USA) when the fragments were above 400 bp or directly spun out of the gel when the fragments were shorter than 400 bp, as described by [Heery et al., TIBS 6 (6), 173 (1990)].

Oligos used for gene amplification and site directed mutagenesis:

All PCR reactions performed to allow the construction of the different plasmids are described below. All the primers used are summarized in figure 14.

Primers #100 and #101 were used in a PCR reaction to amplify the complete *crtE* gene having a *SpeI* restriction site and an artificial ribosomal binding site (RBS) upstream of the transcription start site of this gene. At the 3' end of the amplified fragment, two unique restriction sites were introduced, an *AvrII* and a *SmaI* site, to facilitate the further cloning steps. The PCR reaction was done with UITma polymerase using the following conditions for the amplification: 5 cycles with the profile: 95°C, 1 min./ 60°C, 45 sec./ 72°C, 1 min. and 20 cycles with the profile: 95°C, 1 min./ 72°C, 1 min.. Plasmid pBIKS(+)-clone2 served as template DNA. The final PCR product was digested with *SpeI* and *SmaI* and isolated using the GENECLEAN KIT. The size of the fragment was approx. 910 bp.

Primers #104 and #105 were used in a PCR reaction to amplify the *crtZ* gene from the translation start till the *Sall* restriction site, located in the coding sequence of this gene. At the 5' end of the *crtZ* gene an *EcoRI*, a synthetic RBS and a *NdeI* site was introduced. The PCR conditions were as described above. Plasmid pBIKS(+)-clone 6a served as template DNA and the final PCR product was digested with *EcoRI* and *Sall*. Isolation of the fragment of approx. 480 bp was done with the GENECLEAN KIT.

Primers MUT1 and MUT5 were used to amplify the complete *crtY* gene. At the 5' end, the last 23 nucleotides of the *crtZ* gene including the *Sall* site are present, followed by an artificial RBS preceding the translation start site of the *crtY* gene. The artificial RBS created includes a *PmlI* restriction site. The 3' end of the amplified fragment contains 22 nucleotides of the *crtI* gene, preceded by an newly created artificial RBS which contains a *MunI* restriction site. The conditions used for the PCR reaction were as described above using the following cycling profile: 5 rounds of 95°C, 45 sec./ 60°C, 45 sec./ 72°C, 75 sec. followed by 22 cycles with the profile: 95°C, 45 sec./ 66°C, 45 sec./ 72°C, 75 sec.. Plasmid

pXI12-ZYIB-EINV4 served as template for the Pfu Vent polymerase. The PCR product of 1225 bp was made blunt and cloned into the SmaI site of pUC18, using the Sure-Clone Kit (Pharmacia) according to the manufacturer.

Primers **MUT2** and **MUT6** were used to amplify the complete *crtI* gene. At the 5' the last 23 nucleotides of the *crtY* gene are present, followed by an artificial RBS which precedes the translation start site of the *crtI* gene. The new RBS created, includes a MunI restriction site. The 3' end of the amplified fragment contains the artificial RBS upstream of the *crtB* gene including a BamHI restriction site. The conditions used for the PCR reaction were basically as described above including the following cycling profile: 5 rounds of 95°C, 30 sec./ 60°C, 30 sec./ 72°C, 75 sec., followed by 25 cycles with the profile: 95°C, 30 sec./ 66°C, 30 sec./ 72°C, 75 sec.. Plasmid pXI12-ZYIB-EINV4 served as template for the Pfu Vent polymerase. For the further cloning steps the PCR product of 1541 bp was digested with MunI and BamHI.

Primers **MUT3** and **CAR17** were used to amplify the N-terminus of the *crtB* gene. At the 5' the last 28 nucleotides of the *crtI* gene are present followed by an artificial RBS, preceding the translation start site of the *crtB* gene. This new created RBS, includes a BamHI restriction site. The amplified fragment, named PCR-F contains also the HindIII restriction site located at the N-terminus of the *crtB* gene. The conditions used for the PCR reaction were as described elsewhere in the text, including the following cycling profile: 5 rounds of 95°C, 30 sec./ 58°C, 30 sec./ 72°C, 20 sec. followed by 25 cycles with the profile: 95°C, 30 sec./ 60°C, 30 sec./ 72°C, 20 sec.. Plasmid pXI12-ZYIB-EINV4 served as template for the Pfu Vent polymerase. The PCR product of approx. 160 bp was digested with BamHI and HindIII.

Oligos used to amplify the chloramphenicol resistance gene (*cat*):

Primers **CAT3** and **CAT4** were used to amplify the chloramphenicol resistance gene of pC194 (ATCC 37034) [Hori-nouchi and Weisblum, s.a.] a R-plasmid found in *S. aureus*. The conditions used for the PCR reaction were as described previously including the following cycling profile: 5 rounds of 95°C, 60 sec./ 50°C, 60 sec./ 72°C, 2 min. followed by 20 cycles with the profile: 95°C, 60 sec./ 60°C, 60 sec./ 72°C, 2 min.. Plasmid pC198 served as template for the Pfu Vent polymerase. The PCR product of approx. 1050 bp was digested with EcoRI and AatII.

Oligos used to generate linkers: Linkers were obtained by adding 90 ng of each of the two corresponding primers into an Eppendorf tube. The mixture was dried in a speed vac and the pellet resuspended in 1x Ligation buffer (Boehringer, Mannheim, Germany). The solution was incubated at 50°C for 3 min. before cooling down to RT, to allow the primers to hybridize properly. The linker were now ready to be ligated into the appropriate sites. All the oligos used to generate linkers are shown in figure 15.

Primers **CS1** and **CS2** were used to form a linker containing the following restrictions sites HindIII, AflII, ScaI, XbaI, Pml and EcoRI.

Primers **MUT7** and **MUT8** were used to form a linker containing the restriction sites Sall, AvrII, PmlI, MluI, MunI, BamHI, SphI and HindIII.

Primers **MUT9** and **MUT10** were used to introduce an artificial RBS upstream of *crtY*.

Primers **MUT11** and **MUT12** were used to introduce an artificial RBS upstream of *crtE*.

Isolation of RNA: Total RNA was prepared from log phase growing *B. subtilis* according to the method described by [Maes and Messens, Nucleic Acids Res. 20 (16), 4374 (1992)].

Northern Blot analysis: For hybridization experiments up to 30 µg of *B. subtilis* RNA was electrophoreses on a 1% agarose gel made up in 1x MOPS and 0.66 M formaldehyde. Transfer to Zeta-Probe blotting membranes (BIO-RAD), UV cross-linking, pre-hybridization and hybridization was done as described elsewhere in [Farrell, J.R.E., RNA Methodologies. A laboratory Guide for isolation and characterization. San Diego, USA: Academic Press (1993)]. The washing conditions used were: 2 x 20 min. in 2xSSPE/0.1% SDS followed by 1 x 20min. in 0.1% SSPE/0.1% SDS at 65°C. Northern blots were then analyzed either by a Phosphorimager (Molecular Dynamics) or by autoradiography on X-ray films from Kodak.

Isolation of genomic DNA: *B. subtilis* genomic DNA was isolated from 25 ml overnight cultures according to the standard procedure method 2.6 described by [13].

Southern blot analysis: For hybridization experiments *B. subtilis* genomic DNA (3 µg) was digested with the appropriate restriction enzymes and electrophoresed on a 0.75% agarose gel. The transfer to Zeta-Probe blotting membranes (BIO-RAD), was done as described [Southern, E.M., s.a.]. Prehybridization and hybridization was in 7%SDS, 1% BSA (fraction V; Boehringer), 0.5M Na₂HPO₄, pH 7.2 at 65°C. After hybridization the membranes were washed twice for 5 min. in 2x SSC, 1% SDS at room temperature and twice for 15 min. in 0.1% SSC, 0.1% SDS at 65°C. Southern blots were then analyzed either by a Phosphorimager (Molecular Dynamics) or by autoradiography on X-ray films from Kodak.

DNA sequence analysis: The sequence was determined by the dideoxy chain termination technique [Sanger et al., s.a.] using the Sequenase Kit Version 1.0 (United States Biochemical). Sequence analysis were done using the GCG sequence analysis software package (Version 8.0) by Genetics Computer, Inc. [Devereux et al., s.a.].

Gene amplification in *B. subtilis*: To amplify the copy number of the SFCO in *B. subtilis* transformants, a single colony was inoculated in 15 ml VY-medium supplemented with 1.5 % glucose and 0.02 mg chloramphenicol or neomycin/ml, dependend on the antibiotic resistance gene present in the amplifiable structure (see results and discussion).

The next day 750 µl of this culture were used to inoculate 13 ml VY-medium containing 1.5% glucose supplemented with (60, 80, 120 and 150 µg/ml) for the cat resistant mutants, or 160 µg/ml and 180 µg/ml for the neomycin resistant mutants). The cultures were grown overnight and the next day 50 µl of different dilutions (1: 20, 1:400, 1: 8000, 1: 160'000) were plated on VY agar plates with the appropriate antibiotic concentration. Large single colonies were then further analyzed to determine the number of copies and the amount of carotenoids produced.

Analysis of carotenoids: *E. coli* or *B. subtilis* transformants (200 - 400 ml) were grown for the times indicated in the text, usually 24 to 72 hours, in LB-medium or VY-medium, respectively, supplemented with antibiotics, in shake flasks at 37° C and 220 rpm.

The carotenoids produced by the microorganisms were extracted with an adequate volume of acetone using a rotation homogenizer (Polytron, Kinematica AG, CH-Luzern). The homogenate was filtered through the sintered glass of a suction filter into a round bottom flask. The filtrate was evaporated by means of a rotation evaporator at 50° C using a water-jet vacuum. For the zeaxanthin detection the residue was dissolved in n-hexane/acetone (86:14) before analysis with a normalphase HPLC as described in [Weber, S., s.a.]. For the detection of β-carotene and lycopene the evaporated extract was dissolved in n-hexane/acetone (99:1) and analysed by HPLC as described in Hengartner et al., s.a.].

Example 4

Carotenoid production in *E. coli*

The biochemical assignment of the gene products of the different open reading frames (ORF'S) of the carotenoid biosynthesis cluster of *Flavobacterium sp.* were revealed by analyzing the carotenoid accumulation in *E. coli* host strains, transformed with plasmids carrying deletions of the *Flavobacterium sp.* gene cluster, and thus lacking some of the crt gene products. Similar functional assays in *E. coli* have been described by other authors [Misawa et al., s.a.; Perry et al., J. Bacteriol., 168, 607-612 (1986); Hundle, et al., Molecular and General Genetics 254 (4), 406-416 (1994)]. Three different plasmid pLyco, pBIIKS(+)-clone59-2 and pZea4 were constructed from the three genomic isolates pBIIKS(+)-clone2, pBIIKS(+)-clone59 and pBIIKS(+)-clone6a (see figure 16).

Plasmid pBIIKS(+)-clone59-2 was obtained by subcloning the HindIII/BamHI fragment of pBIIKS(+)-clone 2 into the HindIII/BamHI sites of pBIIKS(+)-clone59. The resulting plasmid pBIIKS(+)-clone59-2 carries the complete ORF's of the crtE, crtB, crtI and crtY gene and should lead to the production of β-carotene. pLyco was obtained by deleting the KpnI/KpnI fragment, coding for approx. one half (N-terminus) of the crtY gene, from the plasmid pBIIKS(+)-clone59-2. *E. coli* cells transformed with pLyco, and therefore having a truncated non-functional crtY gene, should produce lycopene, the precursor of β-carotene. pZea4 was constructed by ligation of the AscI-SpeI fragment of pBIIKS(+)-clone59-2, containing the crtE, crtB, crtI and most of the crtY gene with the AscI/XbaI fragment of clone 6a, containing the crtZ gene and sequences to complete the truncated crtY gene mentioned above. pZea4 has therefore all five ORF's of the zeaxanthin biosynthesis pathway. *E. coli* cells transformed with this latter plasmid should therefore produce zeaxanthin. For the detection of the carotenoid produced, transformants were grown for 43 hours in shake flasks and then subjected to carotenoid analysis as described in the methods section. Figure 16 summarizes the construction of the plasmids described above.

As expected the pLyco carrying *E. coli* cells produced lycopene, those carrying pBIIKS(+)-clone59-2 produced β-carotene (all-E,9-Z,13-Z) and the cells having the pZea4 construct produced zeaxanthin. This confirms that we have cloned all the necessary genes of *Flavobacterium sp.* R1534 for the synthesis of zeaxanthin or their precursors (phytoene, lycopene and β-carotene). The production levels obtained are shown in table 1.

plasmid	host	zeaxanthin	β -carotene	lycopene
pLyco	<i>E. coli</i> JM109	ND	ND	0.05%
pBIIKS(+)-clone59-2	"	ND	0.03%	ND
pZea4	"	0.033%	0.0009%	ND

Table 1: Carotenoid content of *E. coli* transformants, carrying the plasmids pLyco, pBIIKS(+)-clone59-2 and pZea4, after 43 hours of culture in shake flasks. The values indicated show the carotenoid content in % of the total dry cell mass (200 ml). ND = not detectable.

Examples 5

Carotenoid production in *B. subtilis*

In a first approach to produce carotenoids in *B. subtilis*, we cloned the carotenoid biosynthesis genes of *Flavobacterium* into the Gram (+)/(-) shuttle vectors p602/22, a derivative of p602/20 [LeGrice, S.F.J., s.a.]. The assembling of the final construct p602-CARVEG-E, begins with a triple ligation of fragments PvuII-AvrII of pZea4(del654-3028) and the AvrII-EcoRI fragment from plasmid pBIIKS(+)-clone6a, into the EcoRI and ScaI sites of the vector p602/22. The plasmid pZea4(del654-3028) had been obtained by digesting pZea4 with SacI and EspI. The protruding and recessed ends were made blunt with Klenow enzyme and religated. Construct pZea4(del654-3028) lacks most of the sequence upstream of crtE gene, which are not needed for the carotenoid biosynthesis. The plasmid p602-CAR has approx. 6.7 kb of genomic *Flavobacterium R1534* DNA containing besides all five carotenoid genes (approx. 4.9 kb), additional genomic DNA of 1.2 kb, located upstream of the crtZ translation start site and further 200 bp, located upstream of crtE transcription start. The crtZ, crtY, crtI and crtB genes were cloned downstream of the P_{N25/0} promoter, a regulatable *E. coli* bacteriophage T5 promoter derivative, fused to a lac operator element, which is functional in *B. subtilis* [LeGrice, S.F.J., s.a.]. It is obvious that in the p602CAR construct, the distance of over 1200 bp between the P_{N25/0} promoter and the transcription start site of crtZ is not optimal and will be improved at a later stage. An outline of the p602CAR construction is shown in figure 17. To ensure transcription of the crtE gene in *B. subtilis*, the vegI promoter [Moran et al., Mol. Gen. Genet. 186, 339-346 (1982); LeGrice et al., Mol. Gen. Genet. 204, 229-236 (1986)] was introduced upstream of this gene, resulting in the plasmid construct p602-CARVEG-E. The vegI promoter, which originates from siteI of the veg promoter complex described by [LeGrice et al., s.a.] has been shown to be functional in *E. coli* [Moran et al., s.a.]. To obtain this new construct, the plasmid p602CAR was digested with SalI and HindIII, and the fragment containing the complete crtE gene and most of the crtB coding sequence, was subcloned into the XhoI and HindIII sites of plasmid p205. The resulting plasmid p205CAR contains the crtE gene just downstream of the PvegI promoter. To reconstitute the carotenoid gene cluster of *Flavobacterium* sp. the following three pieces were isolated: PmeI/HindIII fragment of p205CAR, the HincII/XbaI fragment and the EcoRI/HindIII fragment of p602CAR and ligated into the EcoRI and XbaI sites of pBluescriptIIKS(+), resulting in the construct pBIIKS(+)-CARVEG-E. Isolation of the EcoRI-XbaI fragment of this latter plasmid and ligation into the EcoRI and XbaI sites of p602/22 gives a plasmid similar to p602CAR but having the crtE gene driven by the PvegI promoter. All the construction steps to get the plasmid p602CARVEG-E are outlined in figure 18. *E. coli* TG1 cells transformed with this plasmid synthesized zeaxanthin. In contrast *B. subtilis* strain 1012 transformed with the same constructs did not produce any carotenoids. Analysis of several zeaxanthin negative *B. subtilis* transformants always revealed, that the transformed plasmids had undergone severe deletions. This instability could be due to the large size of the constructs.

In order to obtain a stable construct in *B. subtilis*, the carotenoid genes were cloned into the Gram (+)/(-) shuttle vector PHP13 constructed by [Haima et al., s.a.]. The stability problems were thought to be omitted by 1) reducing the

size of the cloned insert which carries the carotenoid genes and 2) reversing the orientation of the crtE gene and thus only requiring one promoter for the expression of all five genes, instead of two, like in the previous constructs. Furthermore, the ability of cells transformed by such a plasmid carrying the synthetic *Flavobacterium* carotenoid operon (SFCO), to produce carotenoids, would answer the question if a modular approach is feasible. Figure 19 summarizes all the construction steps and intermediate plasmids made to get the final construct pHP13-2PNZYIB-EINV. Briefly: To facilitate the following constructions, a vector pHP13-2 was made, by introducing a synthetic linker obtained with primer CS1 and CS2, between the HindIII and EcoRI sites of the shuttle vector pHP13. The intermediate construct pHP13-2CARVEG-E was constructed by subcloning the AflII-XbaI fragment of p602CARVEG-E into the AflII and XbaI sites of pHP13-2. The next step consisted in the inversion of crtE gene, by removing XbaI and AvrII fragment containing the original crtE gene and replacing it with the XbaI-AvrII fragment of plasmid pBIISK(+)-PCRRBSctE. The resulting plasmid was named pHP13-2CARZYIB-EINV and represented the first construction with a functional SFCO. The intermediate construct pBIISK(+)-PCRRBSctE mentioned above, was obtained by digesting the PCR product generated with primers #100 and #101 with SpeI and SmaI and ligating into the SpeI and SmaI sites of pBluescriptIIKS(+). In order to get the crtZ transcription start close to the promoter P_{N25/0} a triple ligation was done with the BamHI-Sall fragment of pHP13-2CARZYIB-EINV (contains four of the five carotenoid genes), the BamHI-EcoRI fragment of the same plasmid containing the P_{N25/0} promoter and the EcoRI-Sall fragment of pBIISK(+)-PCRRBSctZ, having most of the crtZ gene preceded by a synthetic RBS. The aforementioned plasmid pBIISK(+)-PCRRBSctZ was obtained by digesting the PCR product amplified with primers #104 and #105 with EcoRI and Sall and ligating into the EcoRI and Sall sites of pBluescriptIIKS(+). In the resulting vector pHP13-2PN25ZYIB-EINV, the SFCO is driven by the bacteriophage T5 promoter P_{N25/0}, which should be constitutively expressed, due to the absence of a functional lac repressor in the construct [Peschke and Beuk, J. Mol. Biol. 186, 547-555 (1985)]. *E. coli* TG1 cells transformed with this construct produced zeaxanthin. Nevertheless, when this plasmid was transformed into *B. subtilis*, no carotenoid production could be detected. Analysis of the plasmids of these transformants showed severe deletions, pointing towards instability problems, similar to the observations made with the aforementioned plasmids.

Examples 6

Chromosome Integration Constructs

Due to the instability observed with the previous constructs we decided to integrate the carotenoid biosynthesis genes of *Flavobacterium* sp. into the genome of *B. subtilis* using the integration/expression vector pX112. This vector allows the constitutive expression of whole operons after integration into the levan-sucrase gene (sacB) of the *B. subtilis* genome. The constitutive expression is driven by the vegI promoter and results in medium level expression. The plasmid pX112-ZYIB-EINV4 containing the synthetic *Flavobacterium* carotenoid operon (SFCO) was constructed as follows: the NdeI-HincII fragment of pBIISK(+)-PCRRBSctZ was cloned into the NdeI and SmaI sites of pX112 and the resulting plasmid was named pX112-PCRctZ. In the next step, the BstEII-PmeI fragment of pHP13-2PN25ZYIB-EINV was ligated to the BstEII-PmeI fragment of pX112-PCRctZ (see figure 20). *B. subtilis* transformed with the resulting construct pX112-ZYIB-EINV4 can integrate the CAR genes either via a Campbell type reaction or via a reciprocal recombination. One transformant, BS1012::ZYIB-EINV4, having a reciprocal recombination of the carotenoid biosynthesis genes into the levan-sucrase gene was further analyzed (figure 21). Although this strain did not synthesize carotenoids, RNA analysis by Northern blots showed the presence of specific polycistronic mRNA's of 5.4 kb and 4.2 kb when hybridized to probe A (see figure 21, panel B). Whereas the larger mRNA has the expected message size, the origin of the shorter mRNA was unclear. Hybridization of the same Northern blot to probe B only detected the large mRNA fragment, pointing towards a premature termination of the transcription at the end of the crtB gene. The presence of a termination signal at this location would make sense, since in the original operon organisation in the *Flavobacterium* sp. R1534 genome, the crtE and the crtB genes are facing each other. With this constellation a transcription termination signal at the 5' end of crtB would make sense, in order to avoid the synthesis of anti-sense RNA which could interfere with the mRNA transcript of the crtE gene. Since this region has been changed considerably with respect to the wild type situation, the sequences constituting this terminator may also have been altered resulting in a "leaky" terminator. Western blot analysis using antisera against the different crt-enzymes of the carotenoid pathway, pointed towards the possibility that the ribosomal binding sites might be responsible for the lack of carotenoid synthesis. Out of the five genes introduced only the product of crtZ, the β -carotene hydroxylase was detectable. This is the only gene preceded by a RBS site, originating from the pX112 vector, known to be functional in *B. subtilis*. Base pairing interactions between a mRNA's Shine-Dalgarno sequence [Shine and Dalgarno, s. a.] and the 16S rRNA, which permits the ribosome to select the proper initiation site, have been proposed by [McLaughlin et al., J. Biol. Chem. 256, 11283-11291 (1981)] to be much more stable in Gram-positive organisms (*B. subtilis*) than in Gram-negative organisms (*E. coli*). In order to obtain highly stable complexes we exchanged the RBS sites of the Gram-negative *Flavobacterium* sp., preceding each of the genes crtY, crtI, crtB and crtE, with synthetic RBS's which were designed complementary to the 3' end of the *B. subtilis* 16S rRNA (see table 2). This exchange should allow an effective translation initiation of the different

carotenoid genes in *B. subtilis*. The strategy chosen to construct this pXI12-ZYIB-EINV4MUTRBS2C, containing all four altered sites is summarized in figure 20. In order to facilitate the further cloning steps in pBluescriptIIKS(+), additional restriction sites were introduced using the linker obtained with primer MUT7 and MUT8, cloned between the Sall and HindIII sites of said vector. The new resulting construct pBIKS(+)-LINKER78 had the following restriction sites introduced: AvrII, PmlI, MuiI, MunI, BamHI and SphI. The general approach chosen to create the synthetic RBS's upstream of the different carotenoid genes, was done using a combination of PCR based mutagenesis, where the genes were reconstructed using defined primers carrying the modified RBS sites, or using synthetic linkers having such sequences. Reconstitution of the RBS preceding the crtI and crtB genes was done by amplifying the crtI gene with the primers MUT2 and MUT6, which include the appropriate altered RBS sites. The PCR-I fragment obtained was digested with MunI and BamHI and ligated into the MunI and BamHI sites of pBIKS(+)-LINKER78. The resulting intermediate construct was named pBIKS(+)-LINKER78PCR-I. Reconstitution of the RBS preceding the crtB gene was done using a small PCR fragment obtained with primer MUT3, carrying the altered RBS site upstream of crtB, and primer CAR17. The amplified PCR-F fragment was digested with BamHI and HindIII and sub cloned into the BamHI and HindIII sites of pBIKS(+)-LINKER78, resulting in the construct pBIKS(+)-LINKER78PCR-F. The PCR-I fragment was cut out of pBIKS(+)-LINKER78PCR-I with BamHI and SapI and ligated into the BamHI and SapI sites of pBIKS(+)-LINKER78PCR-F. The resulting plasmid pBIKS(+)-LINKER78PCR-FI has the PCR-I fragment fused to the PCR-F fragment. This construct was cut with Sall and PmlI and a synthetic linker obtained by annealing of primer MUT9 and MUT10 was introduced. This latter step was done to facilitate the upcoming replacement of the original *Flavobacterium* RBS in the above mentioned construct. The resulting plasmid was named pBIKS(+)-LINKER78PCR-FIA. Assembling of the synthetic RBS's preceding the crtY and crtI genes was done by PCR, using primers MUT1 and MUT5. The amplified fragment PCR-G was made blunt end before cloning into the SmaI site of pUC18, resulting in construct pUC18-PCR-G. The next step was the cloning of the PCR-G fragment between the PCR-A and PCR-I fragments. For this purpose the PCR-G was isolated from pUC18-PCR-G by digesting with MunI and PmlI and ligated into the MunI and PmlI sites of pBIKS(+)-LINKER78PCR-FIA. This construct contains all four fragments, PCR-F, PCR-I, PCR-G and PCR-A, assembled adjacent to each other and containing three of the four artificial RBS sites (crtY, crtI and crtB). The exchange of the *Flavobacterium* RBS's preceding the genes crtY, crtI and crtB by synthetic ones, was done by replacing the HindIII-Sall fragment of plasmid pXI12-ZYIB-EINV4 with the HindIII-Sall fragment of plasmid pBIKS(+)-LINKER78PCR-FIA. The resulting plasmid pXI12-ZYIB-EINV4 MUTRBS2C was subsequently transformed into *E. coli* TG1 cells and *B. subtilis* 1012. The production of zeaxanthin by these cells confirmed that the PCR amplified genes were functional. The *B. subtilis* strain obtained was named BS1012::SFCO1. The last *Flavobacterium* RBS to be exchanged was the one preceding the crtE gene. This was done using a linker obtained using primer MUT11 and MUT12. The wild type RBS was removed from pXI12-ZYIB-EINV4MUTRBS2C with NdeI and SpeI and the above mentioned linker was inserted. In the construct pXI12-ZYIB-EINV4MUTRBS2C all *Flavobacterium* RBS's have been replaced by synthetic RBS's of the consensus sequence AAAGGAGG-7-8 N-ATG (see table 2). *E. coli* TG1 cells transformed with this construct showed that also this last RBS replacement had not interfered

Table 2

mRNA	nucleotide sequence
crtZ	AAAGGAGGGUUUCAU <u>AUG</u> AGC
crtY	AAAGGAGGACACGUGA <u>AUG</u> AGC
crtI	AAAGGAGGCAAUUGAGA <u>AUG</u> AGU
crtB	AAAGGAGGAUCCAAUCA <u>AUG</u> ACC
crtE	AAAGGAGGGUUUCUU <u>AUG</u> ACG

<i>B. subtilis</i>	16S rRNA	3'-UC UUUUCCUCC ACUAG
<i>E. coli</i>	16S rRNA	3'-AUUCCUCCACUAG

Table 2: Nucleotide sequences of the synthetic ribosome binding sites in the constructs pXI12-ZYIB-EINV4MUTRBS2C, pXI12-ZYIB-EINV4MUTRBS2CCAT and pXI12-ZYIB-EINV4MUTRBS2CNEO. Nucleotides of the Shine-Dalgarno sequence preceding the individual carotenoid genes which are complementary to the 3' ends of the 16S rRNA of *B. subtilis* are shown in bold. The 3' ends of the 16S rRNA of *E. coli* is also shown as comparison. The underlined AUG is the translation start site of the mentioned gene.

with the ability to produce zeaxanthin. All the regions containing the newly introduced synthetic RBS's were confirmed by sequencing. *B. subtilis* cells were transformed with plasmid pXI12-ZYIB-EINV4MUTRBS2 and one transformant having integrated the SFCO by reciprocal recombination, into the levan-sucrase gene of the chromosome, was selected. This strain was named BS1012::SFCO2. Analysis of the carotenoid production of this strain show that the amounts zeaxanthin produced is approx. 40% of the zeaxanthin produced by *E. coli* cells transformed with the plasmid used to get the *B. subtilis* transformant. Similar was the observation when comparing the BS1012::SFCO1 strain with its *E. coli* counter part (30%). Although the *E. coli* cells have 18 times more carotenoid genes, the carotenoid production is only a factor of 2-3 times higher. More drastic was the difference observed in the carotenoid contents, between *E. coli* cells carrying the pZea4 construct in about 200 copies and the *E. coli* carrying the plasmid pXI12-ZYIB-EINV4MUTRBS2C in 18 copies. The first transformant produced 48x more zeaxanthin than the latter one. This difference seen can not only be attributed to the roughly 11 times more carotenoid biosynthesis genes present in these transformants. Contributing to this difference is probably also the suboptimal performance of the newly constructed SFCO, in which the overlapping genes of the wild type *Flavobacterium* operon were separated to introduce the synthetic RBS's. This could have resulted in a lower translation efficiency of the rebuild synthetic operon (e.g. due to elimination of putative translational coupling effects, present in the wild type operon).

In order to increase the carotenoid production, two new constructs were made, pXI12-ZYIB-EINV4MUTRBS2CNEO and pXI12-ZYIB-EINV4MUTRBS2CCAT, which after the integration of the SFCO into the levan-sucrase site of the chromosome, generate strains with an amplifiable structure as described by [Janniere et al., Gene 40, 47-55 (1985)]. Plasmid pXI12-ZYIB-EINV4MUTRBS2CNEO has been deposited on May 25, 1995 at the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany) under accession No. DSM 10013. Such amplifiable structures, when linked to a resistance marker (e.g. chloramphenicol, neomycin, tetracycline), can be amplified to 20-50 copies per chromosome. The amplifiable structure consist of the SFCO, the resistance gene and the pXI12 sequence, flanked by direct repeats of the sac-B 3' gene (see figure 22). New strains having elevated numbers of the SFCO could now be obtained by selecting for transformants with increased level of resistance to the antibiotic. To construct plasmid pXI12-ZYIB-EINV4MUTRBS2CNEO, the neomycin resistance gene was isolated from plasmid pBEST501 with PstI and SmaI and subcloned into the PstI and EcoO1091 sites of the pUC18 vector. The resulting construct was named pUC18-Neo. To get the final construct, the PmeI - AatII fragment of plasmid pXI12-ZYIB-EINV4MUTRBS2C was replaced with the SmaI-AatII fragment of pUC18-Neo, containing the neomycin resistance gene. Plasmid pXI12-ZYIB-EINV4MUTRBS2CCAT was obtained as follows: the chloramphenicol resistance gene of pC194 was isolated by PCR using the primer pair cat3 and cat4. The fragment was digested with EcoRI and AatII and subcloned into the EcoRI and AatII sites of pUC18. The resulting plasmid was named pUC18-CAT. The final vector was obtained by replacing the PmeI-AatII fragment of pXI12-ZYIB-EINV4MUTRBS2C with the EcoRI-AatII fragment of pUC18-CAT, carrying the chloramphenicol resistance gene. Figure 23 summarizes the different steps to obtain aforementioned constructs. Both plasmids were transformed into *B. subtilis* strain 1012, and transformants resulting from a

Campbell-type integration were selected. Two strains BS1012::SFCONEO1 and BS1012::SFCOCAT1 were chosen for further amplification. Individual colonies of both strains were independently amplified by growing them in different concentrations of antibiotics as described in the methods section. For the cat gene carrying strain, the chloramphenicol concentrations were 60, 80, 120 and 150 µg/ml. For the neo gene carrying strain, the neomycin concentrations were 160 and 180 µg/ml. In both strains only strains with minor amplifications of the SFCO's were obtained. In daughter strains generated from strain BS1012::SFCONEO1, the resistance to higher neomycin concentrations correlated with the increase in the number of SFCO's in the chromosome and with higher levels of carotenoids produced by these cells. A different result was obtained with daughter strains obtained from strain BS1012::SFCOCAT1. In these strains an increase up to 150 µg chloramphenicol/ml resulted, as expected, in a higher number of SFCO copies in the chromosome.

Example 7

Construction of CrtW containing plasmids and use for carotenoid production

Polymerase chain reaction based gene synthesis. The nucleotide sequence of the artificial crtW gene, encoding the β-carotene β-4-oxygenase of *Alcaligenes* strain PC-1, was obtained by back translating the amino acid sequence outlined in [Misawa, 1995], using the BackTranslate program of the GCG Wisconsin Sequence Analysis Package, Version 8.0 (Genetics Computer Group, Madison, WI, USA) and a codon frequency reference table of *E. coli* (supplied by the Bach Translate Program). The synthetic gene consisting of 726 nucleotides was constructed basically according to the method described by [Ye, 1992]. The sequence of the 12 oligonucleotides (crtW1 - crtW12) required for the synthesis are shown in Figure 25. Briefly, the long oligonucleotides were designed to have short overlaps of 15-20 bases, serving as primers for the extension of the oligonucleotides. After four cycles a few copies of the full length gene should be present which is then amplified by the two terminal oligonucleotides crtW15 and crtW26. The sequences for these two short oligonucleotides are for the forward primer crtW15 (5'-TATATCTAGAcataTCCGGTCGTAAA CCGG-3') and for the reverse primer crtW26 (5'-TATAgaaattccacgtTCA AGCACGACCACCGGTTTTACG-3'), where the sequences matching the DNA templates are underlined. Small cap letters show the introduced restriction sites (*Nde*I for the forward primer and *Eco*RI and *Pml*I for the reverse primer) for the latter cloning into the pALTER-Ex2 expression vector.

Polymerase chain reaction. All twelve long oligonucleotides (crtW1-crtW12; 7 nM each) and both terminal primers (crtW15 and crtW26; 0.1 mM each) were mixed and added to a PCR reaction mix containing Expand™ High Fidelity polymerase (Boehringer, Mannheim) (3.5 units) and dNTP's (100 mM each). The PCR reaction was run for 30 cycles with the following profile: 94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min. The PCR reaction was separated on a 1% agarose gel; and the band of approx. 700 bp was excised and purified using the glass beads method (GeneClean Kit, Bio101, Vista, CA, USA). The fragment was subsequently cloned into the *Sma*I site of plasmid pUC18, using the Sure-Clone Kit (Pharmacia, Uppsala, Sweden). The sequence of the resulting crtW synthetic gene was verified by sequencing with the Sequenase Kit Version 1.0 (United States Biochemical, Cleveland, OH, USA). The crtW gene constructed by this method was found to contain minor errors, which were subsequently corrected by site-directed mutagenesis.

Construction of plasmids. Plasmid pBIIKS(+)-CARVEG-E (see also Example 5) (Figure 26) contains the carotenoid biosynthesis genes (crtE, crtB, crtY, crtI and crtZ) of the Gram (-) bacterium *Flavobacterium* sp. strain R1534 WT (ATCC 21588) [Pasamontes, 1995 #732] cloned into a modified pBluescript II KS(+) vector (Stratagene, La Jolla, USA) carrying site I of the *B. subtilis* veg promoter [LeGrice, 1986 #806]. This constitutive promoter has been shown to be functional in *E. coli*. Transformants of *E. coli* strain TG1 carrying plasmid pBIIKS(+)-CARVEG-E synthesise zeaxanthin. Plasmid pALTER-Ex2-crtW was constructed by cloning the *Nde*I - *Eco*RI restricted fragment of the synthetic crtW gene into the corresponding sites of plasmid pALTER-Ex2 (Promega, Madison, WI). Plasmid pALTER-Ex2 is a low copy plasmid with the p15a origin of replication, which allows it to be maintained with ColE1 vectors in the same host. Plasmid pBIIKS-crtEBIYZW (Figure 26) was obtained by cloning the *Hind*III-*Pml*I fragment of pALTER-Ex2-crtW into the *Hind*III and the blunt end made *Mlu*I site obtained by a fill in reaction with Klenow enzyme, as described elsewhere in [Sambrook, 1989 #505]. Inactivation of the crtZ gene was done by deleting a 285 bp *Nsi*I-*Nsi*I fragment, followed by a fill in reaction and religation, resulting in plasmid pBIIKS-crtEBIY[ΔZ]W. Plasmid pBIIKS-crtEBIY[ΔZW] carrying the non-functional genes crtW and crtZ, was constructed by digesting the plasmid pBIIKS-crtEBIY[ΔZ]W with *Nde*I and *Hpa*I, and subsequent self religation of the plasmid after filling in the sites with Klenow enzyme. *E. coli* transformed with this plasmid had a yellow-orange colour due to the accumulation of β-carotene. Plasmid pBIIKS-crtEBIY[ΔW] has a truncated crtW gene obtained by deleting the *Nde*I - *Hpa*I fragment in plasmid pBIIKS-crtEBIYZW as outlined above. Plasmids pALTER-Ex2-crtEBIY[ΔZW] and pALTER-Ex2-crtEBIYZ[ΔW], were obtained by isolating the *Bam*HI-*Xba*I fragment from pBIIKS-crtEBIY[ΔZW] and pBIIKS-crtEBIYZ[ΔW], respectively and cloning them into the *Bam*HI and *Xba*I sites of pALTER-Ex2. The plasmid pBIIKS-crtW was constructed by digesting pBIIKS-crtEBIYZW with *Nsi*I and *Sac*I, and self-religating the plasmid after recessing the DNA overhangs with Klenow enzyme. Figure 27 compiles the relevant inserts of all the plasmids used in this paper.

Carotenoid analysis. *E. coli* TG-1 transformants carrying the different plasmid constructs were grown for 20 hours in Luria-Broth medium supplemented with antibiotics (ampicillin 100 µg/ml, tetracyclin 12.5 µg/ml) in shake flasks at 37°C and 220 rpm. Carotenoids were extracted from the cells with acetone. The acetone was removed in vacuo and the residue was re dissolved in toluene. The coloured solutions were subjected to high-performance liquid chromatog-

raphy (HPLC) analysis which was performed on a Hewlett-Packard series 1050 instrument. The carotenoids were separated on a silica column Nucleosil Si - 100, 200 x 4 mm, 3m. The solvent system included two solvents: hexane (A) and hexane/THF, 1:1 (B). A linear gradient was applied running from 13 to 50 % (B) within 15 minutes. The flow rate was 1.5 ml/min. Peaks were detected at 450 nm by a photo diode array detector. The individual carotenoid pigments were identified by their absorption spectra and typical retention times as compared to reference samples of chemically pure carotenoids, prepared by chemical synthesis and characterised by NMR, MS and UV-Spectra. HPLC analysis of the pigments isolated from *E. coli* cells transformed with plasmid pBIIKS-crtEBIYZW, carrying besides the carotenoid biosynthesis genes of *Flavobacterium* sp. strain R1534, also the crtW gene encoding the β-carotene ketolase of *Alcaligenes* PC-1 [Misawa, 1995 #670] gave the following major peaks identified as: β-cryptoxanthin, astaxanthin, adonixanthin and zeaxanthin, based on the retention times and on the comparison of the absorbance spectra to given reference samples of chemically pure carotenoids. The relative amount (area percent) of the accumulated pigment in the *E. coli* transformant carrying pBIIKS-crtEBIYZW is shown in Table 3 ["CRX": cryptoxanthin; "ASX": astaxanthin; "ADX": adonixanthin; "ZXN": zeaxanthin; "ECM": echinenone; "MECH": 3-hydroxyechinenone, "CXN": cantaxanthin]. The Σ of the peak areas of all identified carotenoids was defined as 100%. Numbers shown in Table 3 represent the average value of four independent cultures for each transformant. In contrast to the aforementioned results, *E. coli* transformants carrying the same genes but on two plasmids namely, pBIIKS-crtEBIYZ[ΔW] and pALTER-Ex2-crtW, showed a drastical drop in adonixanthin and a complete lack of astaxanthin pigments (Table 3), whereas the relative amount of zeaxanthin (%) had increased. Echinenone, hydroxyechinenone and canthaxanthin levels remained unchanged compared to the transformants carrying all the crt genes on the same plasmid (pBIIKS-crtEBIYZΔW). Plasmid pBIIKS-crtEBIYZ[ΔW] is a high copy plasmid carrying the functional genes of crtE, crtB, crtY, crtI, crtZ of *Flavobacterium* sp. strain R1534 and a truncated, non-functional version of the crtW gene, whereas the functional copy of the crtW gene is located on the low copy plasmid pALTER-Ex2-crtW. To analyze the effect of overexpression of the crtW gene with respect to the crtZ gene, *E. coli* cells were co-transformed with plasmid pBIIKS-crtW carrying the crtW gene on the high copy plasmid pBIIKS-crtW and the low copy construct pALTER-Ex2-crtEBIYZ[ΔW], encoding the *Flavobacterium* crt genes. Pigment analysis of these transformants by HPLC monitored the presence of β-carotene, cryptoxanthin, astaxanthin, adonixanthin, zeaxanthin, 3-hydroxyechinenone and minute traces of echinenone and canthaxanthin (Table 3). Transformants harbouring the crtW gene on the low copy plasmid pALTER-Ex2-crtW and the genes crtE, crtB, crtY and crtI on the high copy plasmid pBIIKS-crtEBIYZ[ΔZW] expressed only minor amounts of canthaxanthin (6 %) but high levels of echinenone (94%), whereas cells carrying the crtW gene on the high copy plasmid pBIIKS-crtW and the other crt genes on the low copy construct pALTER-Ex2-crtEBIYZ[ΔZW], had 78.6 % and 21.4 % of echinenone and canthaxanthin, respectively (Table 3).

Table 3

plasmids	CRX	ASX	ADX	ZXN	ECH	HECH	CXN
pBIIKS-crtEBIYZW	1.1	2.0	44.2	52.4	< 1	< 1	< 1
pBIIKS-crtEBIYZ[ΔW] + pALTER-Ex2-crtW	2.2	-	25.4	72.4	< 1	< 1	< 1
pBIIKS-crtEBIYZ[ΔZ]W	-	-	-	-	66.5	-	33.5
pBIIKS-crtEBIYZ[ΔZW] + pBIIKS-crtW	-	-	-	-	94	-	6

Claims

1. A DNA sequence comprising one or more DNA sequences selected from the group consisting of:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous;

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous;

e) a DNA sequence which encodes the β -carotene hydroxylase of *Flavobacterium* sp. R1534 (crtZ) or a DNA sequence which is substantially homologous.

2. A DNA sequence as claimed in claim 1 comprising the following DNA sequences:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous, and

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous, and

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous.

3. A DNA sequence as claimed in claim 1 comprising the following DNA sequences:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous, and

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous, and

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous, and

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous.

4. A DNA sequence as claimed in claim 1 comprising the following DNA sequences:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous, and

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous, and

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous, and

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous, and

e) a DNA sequence which encodes the β -carotene hydroxylase of *Flavobacterium* sp. R1534 (crtZ) or a DNA sequence which is substantially homologous.

5. A DNA sequence as claimed in claim 4 which comprises in addition to the DNA sequences specified in claim 4 a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous.

6. A DNA sequence as claimed in claim 3 which comprises in addition to the DNA sequences specified in claim 3 a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous.

7. A vector comprising the DNA sequence of claim 1.
8. A vector comprising the DNA sequence of claim 2.
- 5 9. A vector comprising the DNA sequence of claim 3.
- 10 10. A vector comprising the DNA sequence of claim 4.
11. A vector comprising the DNA sequence of claim 5.
12. A vector comprising the DNA sequence of claim 6
13. A cell which is transformed by the DNA sequence of claim 1 or the vector of claim 7.
- 15 14. A cell which is transformed by the DNA sequence of claim 2 or the vector of claim 8.
15. A cell which is transformed by the DNA sequence of claim 3 or the vector of claim 9.
16. A cell which is transformed by the DNA sequence of claim 4 or the vector of claim 10.
- 20 17. A cell which is transformed by the DNA sequence of claim 5 or the vector of claim 11.
18. A cell which is transformed by the DNA sequence of claim 6 or the vector of claim 12.
- 25 19. A cell which is transformed by the DNA sequence of claim 4 or the vector of claim 10 and a second DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous or a second vector which comprises a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous.
- 30 20. A cell which is transformed by the DNA sequence of claim 3 or the vector of claim 9 and a second DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous or a second vector which comprises a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous.
- 35 21. The cell of any one of claims 13 to 20 which is a prokaryotic cell.
22. The cell of claim 21 which is *E. coli*.
23. The cell of claim 21 which is a *Bacillus* strain.
- 40 24. The cell of any one of claims 13 to 20 which is an eukaryotic cell.
25. The cell of claim 24 which is a yeast cell.
- 45 26. The cell of claim 24 which is a fungal cell.
27. A process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing a cell as claimed in any one of claims 13 to 26 under suitable culture conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present.
- 50 28. A process as claimed in claim 27 for the preparation of lycopene by culturing a cell as claimed in claim 14.
29. A process as claimed in claim 27 for the preparation of β -carotene by culturing a cell as claimed in claim 15.
- 55 30. A process as claimed in claim 27 for the preparation of echinenone by culturing cells as claimed in claim 18 or 20.
31. A process as claimed in claim 27 for the preparation of canthaxanthin by culturing cells as claimed in claim 18.

32. A process as claimed in claim 27 for the preparation of zeaxanthin by culturing cells as claimed in claim 17 or 19.
33. A process as claimed in claim 27 for the preparation of adonixanthin by culturing cells as claimed in claim 17 or 19.
34. A process as claimed in claim 27 for the preparation of astaxanthin by culturing cells as claimed in claim 17.
35. A process for the preparation of a food or feed composition characterized therein that after a process as claimed in any one of claims 27 to 34 has been effected the carotenoid or carotenoid mixture is added to food or feed.

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Fig. 1

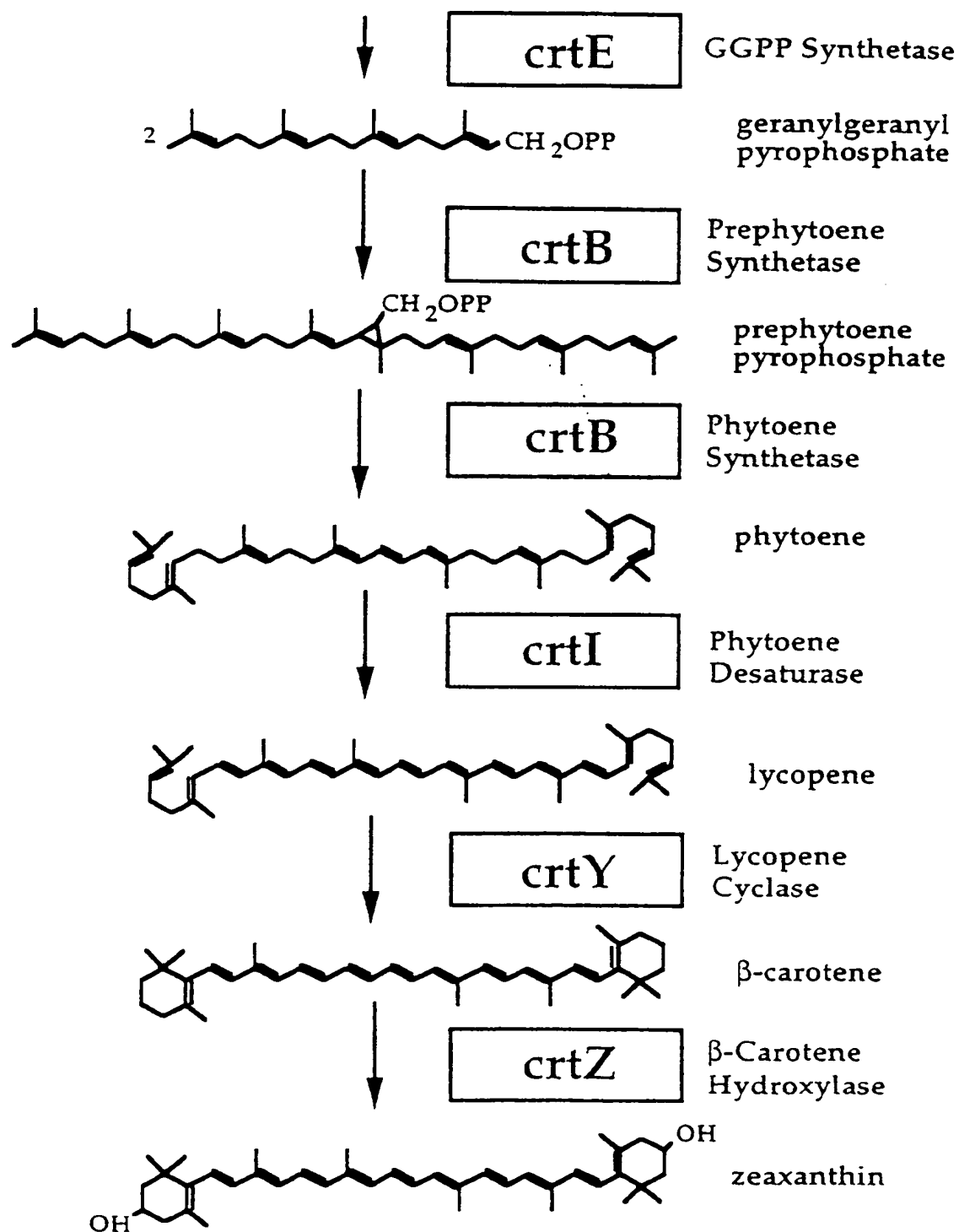


Fig. 2

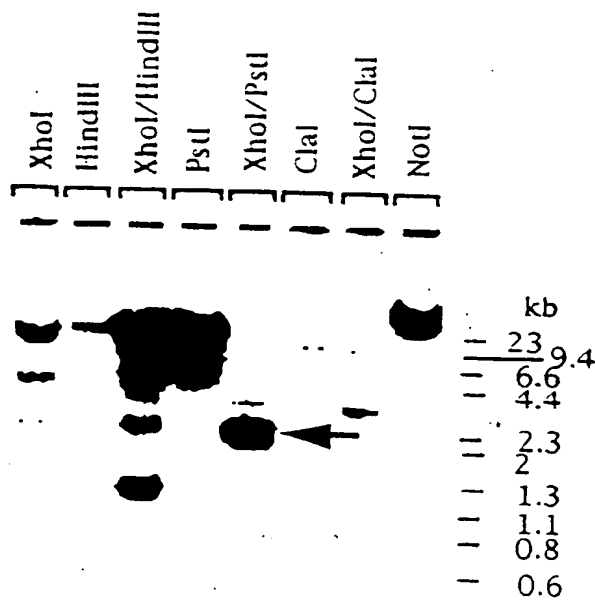


Fig. 3

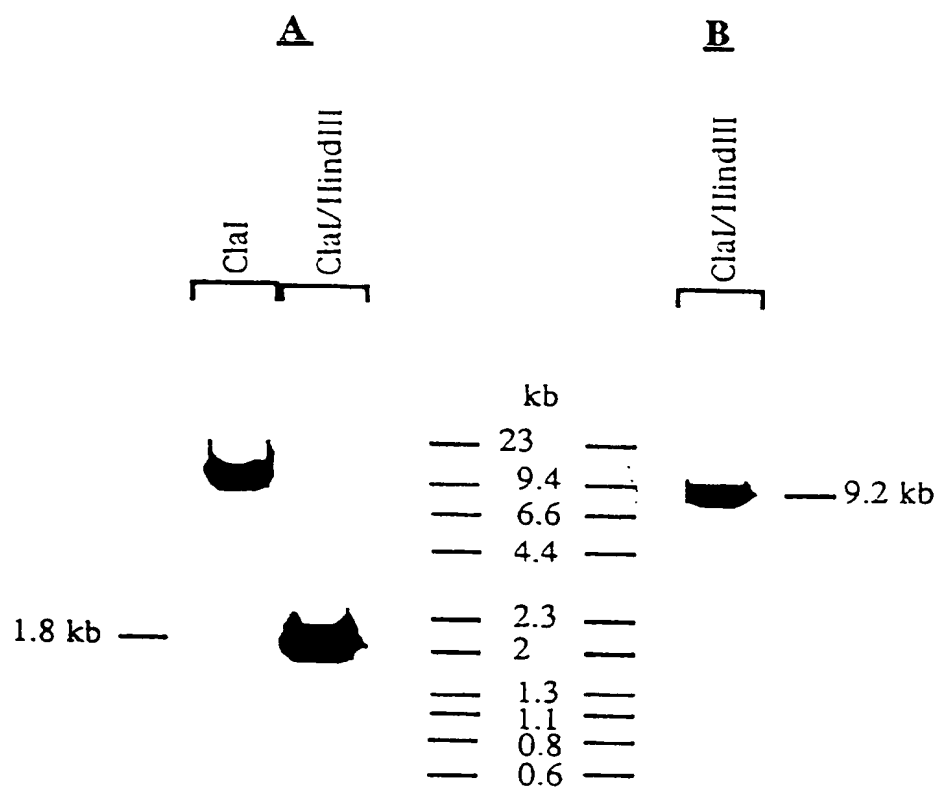


Fig. 4

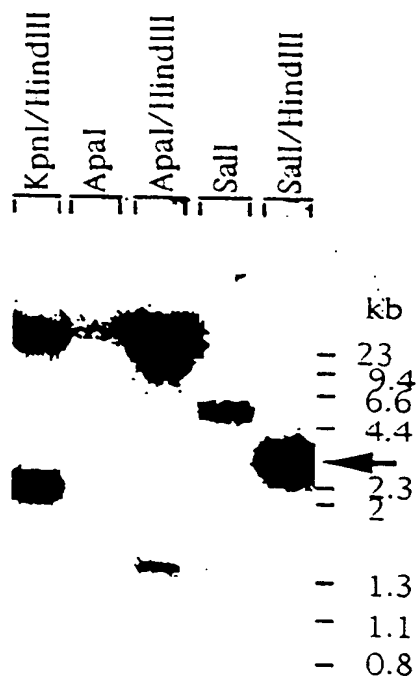


Fig. 5

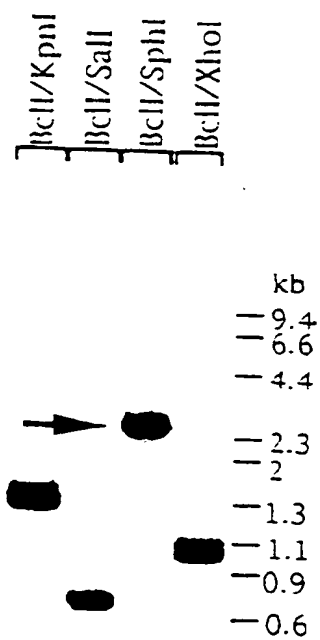


Fig. 6

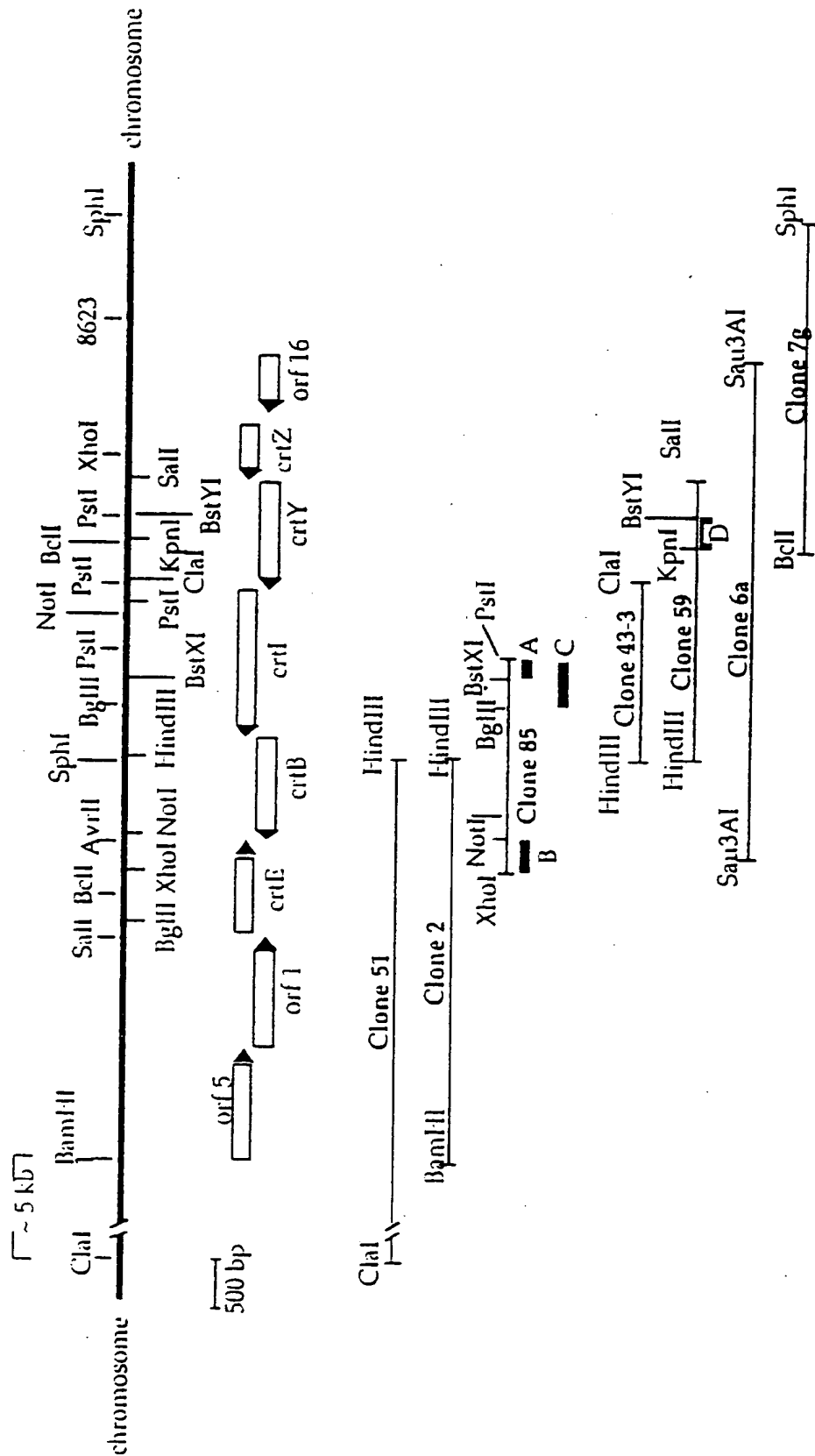


Fig. 7/1

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 C C T A G C G C G A C G G C A G C G C T A G T G T C G C G G G A C G C T A G C C A G
 orf-5 --> D P R L A V R D Q Q P P L R I G Q
 51 A G C A T C A T C C C A T G A C C G C A G C G C A G C A G C G C G C G C C C A G A T C 100
 T C G T A C T A G G G T A C T T G C G C T C G C G T G C G T C G C G C G C G C G C T A G
 H H P H E P Q R T T Q R A P Q I
 101 G G G C G C T C C A G C A G C A T G C G C C A T C A T C G C G A G C C C C G C G G C C A 150
 C C C G C C A G T C G T G C G T A C G C G T A G C G C T T C G C G C G C G C G C G T
 G R V Q H G M R H R E G P R R H
 151 T G G G C G C T G C C A T T C C G A G A C T C G C A G C C T G T C G C T G C G C A G G 200
 A C C C G C C A C G G T A A G G C T T C T T G A C G T G G G A C A G C G C A C G C T T C C
 G A R A H S E E L A A C P L R K V
 201 T C G C C C A G A T C G C G C G T A T T C G A T C C A G T G A C G C C C C C A T C C C G T 250
 A C C G C G T C T A G C G C C A T A G C C T A G C T A C T G C C C G C G C T A C G C C A
 A P D R A V F R C S D G P D A R
 251 G G C C C G C C T G C C C G C C A C C A G G C A T C C G C C A C G A C C C T T C G G 300
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 G P A L P R R H Q R I A H E P F R
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 D D V L I H G P S L Q N R S P I L
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 T G A A S G L G A A S A R M L A Q
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 P E G A V H A A C D V T D A T A A

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-----+-----+-----+-----+-----+-----+-----+
V A G M T L P M A R D L A R H G I
-----+-----+-----+-----+-----+-----+-----+
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-----+-----+-----+-----+-----+-----+-----+
AGCGCATG TACTG TACCGCGCGCGTACAA GCGGTGGGCTAGCACCTC
-----+-----+-----+-----+-----+-----+-----+
R V M T I A P G I F R T P M L E
-----+-----+-----+-----+-----+-----+-----+
GGCGTCCGCA GGA CGTTCA GCA CA GCGTGGCGCGCGCGGTGCCCTTCCC
-----+-----+-----+-----+-----+-----+-----+
CGCA CGCGCTCTCGA AGTCTCTGCGA CCGCGCGCGCGCA CGGAA GGG
-----+-----+-----+-----+-----+-----+-----+
G L P Q D V Q D S L G A A V P F P
-----+-----+-----+-----+-----+-----+-----+
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-----+-----+-----+-----+-----+-----+-----+
S R L G E P S E Y A A L L H H I I
-----+-----+-----+-----+-----+-----+-----+
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R M A P K * M D P I V I T
-----+-----+-----+-----+-----+-----+-----+
orf-1-->

Fig. 7/3

1201	GGCGGATGGCA CCCCGATGGGGGATTCACGGGGGATCTTGGCGGAT -----+-----+-----+-----+-----+ CCGGCTACGGTGGGGTACCCCGTAGGGTCCCGCTAGACGGGGCTA G A M R T P M G A F Q G D L A A N	1250	1501	GTCTGCGCGGGGGATGGAGAGCATGTTCAGAGCGCCCGCTACCTGCTGCC -----+-----+-----+-----+-----+ CAGCA CGGGCGCGCTACCTCTCTGTACAGCTTGGGGGGGATGGACGAGG V V A G G M E S M S N A P Y L L P	1550
1251	GGATGCCCCGACCTTGCGCGGACCGGATCGCGCGCGCGCTGMA CGGCG -----+-----+-----+-----+-----+ CCTACGGGGCTGGGACCGCGCGCTGGGCTAGCGCGGGCGGACTTGGCGG D A P T L G A D A I R A A L N G L	1300	1551	CMA GGGCGGCTCGGGGATGGCGATGGGCCATGACCGCTGTCTGATCA CA -----+-----+-----+-----+-----+ GTTGCGCGCGACCGCTACCGGTACCGGTACTGGCA CAGACCTACTGT K A R S G M R M G H D R V L D H M	1600
1301	TGTCGCGGACATGGTCGACGAGTGTCTGATGGGCTGGTCTCGCGCGG -----+-----+-----+-----+-----+ ACAGCGGGCTGTACCACTGCTCCAGACTACCGGACGCGGAGCGGGCGG S P D M V D E V L M G C V L A A	1350	1601	TGTTCTCGACGGGTTGGA GGCACCTATGACAGGGCGCGCTGATGGGC -----+-----+-----+-----+-----+ ACAGGAGCTGGCCAGCTCTCTGGGGATCTGTTCGCGCGGACTACCCG F L D G L E D A Y D K G R L M G	1650
1351	GGCCAGGCTCAGGCA CGGCA CGTCA GCGCGCGCTTGGCGCGGACTGCC -----+-----+-----+-----+-----+ CGGGTCCAGTCCGTGGCGGTGAGTCCGCGCGGACCGCGGCTGACGG G Q Q Q A P A R Q A A L G A G L P	1400	1651	AGCTTCGCGGAGGATTCGCGCGCGGATCACGGTTTCACCGCGGAGCGCCA -----+-----+-----+-----+-----+ TGGAGCGGCTCTGTACGCGCGCGCTAGTGCAGAGTGGCGCGCTCGCGT T F A E D C A G D H G F T R E A Q	1700
1401	CCTGTGACGGGCA CAGACCATCAAGCAGATGTCCGATCGGGCATGA -----+-----+-----+-----+-----+ CGACAGCTCCCGCTGCTGGTGGTAGTTGCTCTACAGCGCTACCGCTACT L S T G T T T I N E M C G S G M K	1450	1701	GGACGACTATGGCTCA CAGCGCTTGGCGCGCGGCGGCA GCGCATCGCCA -----+-----+-----+-----+-----+ CCTGCTGATACGGGACTGTCTCGACCGCGCGGGTCTCTGCGGTACGGT D D Y A L T S L A R A Q D A I A S	1750
1451	AGCGCGGATGCTGGGCA TGCCTGATCGCGCGGGATCGCGGGGATC -----+-----+-----+-----+-----+ TCCGGGCTACGACCGGCTACTGCATAGCGCGCGCTAGCGCGCGCGTAG A A M L G H D L I A A G S A G I	1500	1751	GGGTGCTTCCCGCGCGGATCGCGCGCGTGA CGGTCACCGCACCGCAAG -----+-----+-----+-----+-----+ CGCCACGAGCGCGCGGCTGTAGCGCGGCACTGCACGTGCGGTGCGTTC G A F A A E I A P V T V T A R K	1800

Fig. 7/4

1801	GTCAGACCAAGCTCGATACGACGAGATGCCGGGCAAGGCGCGCGCGA	1850	2101	TACGACCTGTTGAGGTGAACGAGGCAATTCGCGCTGTCGCCATCATCGC	2150
	CACCTCTGTCGACGCTATGCTGCTTACGGGCGCTTCGCGGCGGGCT			ATGCTGCACAGCTCCACTTGTCTCGGTAAAGCGGCGAGCGAGCGGTACTAGCG	
	V Q T T V D T D E M P G K A R P E			Y D L F E V N E A F A V V A M I A	
1901	GAGATCCCCCATCTGAAGCGCGCTTCCCTGACGGTGCACGGTCAAG	1900	2151	GATGAGGAGCTTGGCTGCCACACGATGCCAGGACATCAACGGCGGG	2200
	CTTGTAGGGGTAGACTTCGGGCGGAGGCACTGCCACCGTGCAGTGGC			CTACTCTCTCGAACCGGAGGCTGTCTACGCTCTTGTAGTTGCGCGCGC	
	K I P H L K P A F R D G G T V T A			M K E L G L P H D A T N I N G G A	
1901	CGGCAACACCTGCTGATCTCGGACGGGCGCGCGCGCTGCTGATGATG	1950	2201	CCTCGCGCTTGGGCAATCCATCGCGCGCTCGCGGCGCGGATCATGCTC	2250
	GCGCTTGTGAGCAGCTAGACGCTGCGCGCGCGCGCGCGCGCTACTAC			GAGCGCGGAAACCGGTAGCGGTAGCGCGGAGCGCGCGCGCTAGTACGAG	
	A N S S I S D G A A A L V M M			C A L G H P I G A S G A R I M V	
1951	CGCAATGACGCGCGAGAGCTGGGCTGACCGCGCATCGCGCGATCAT	2000	2251	ACGCTGCTGAAGCGATGCGCGCGCGCGCGCGCGCGCGCGCGCGATC	2300
	GCGCTCAGCGTCCGCTCTTCGACCGGACTGGGCTAGCGCGCGCTAGTA			TGCGACGACTTGCCTACCGCGCGCGCGCGCGCGCGCGCGCGCGCTAG	
	R Q S Q A E K L G L T P I A R I I			T L L N A M A A R G A T R G A A S	
2001	CGTCAATCCAGCCATGCGGACCGCTCCCGCGCTGTTCCCGACGCGCGCA	2050	2301	CGTCTCATTCGCGCGCGCGAGCGGACGCGCATCGCGCTGCAACGCGCTGA	2350
	GCGAGTACGCTGGGTACCGCTGGGAGGCGCGGACGAGGCGCTGCGGGGT			GCGAGCTAGCGCGCGCGCGCTGCGCGGTAGCGCGGACCTTGCCTACT	
	G H A T H A D R P G L P T A P I			V C I G G G E A T A I A L E R L S	
2051	TGCGCGGATGCGGAGCTGCTGACCGGACGAGCACCGCGCTTGGGAT	2100	2351	GCTAATTCATTTGCGGAAATCGCGCTTTTTCGTGCACGATCGCGGAAACCG	2400
	AGCGCGCTACCGCTTCAACGACTGCGCTGCGCTGCGCTGCGGCGGAGCGCTA			CGATTAAATGAACCGCTTAGCGCGGAAAGCAAGCTACTACCGCGCTGCGC	
	G A M R K L L D R T D T R L G D			*	

Fig. 7/5

2401	GAAGCGCCACGCTGTGTGTTGGTTCGGTCGACCTGTCTTTCGGGCGCATGCC -----+-----+-----+-----+-----+ CTTTGCGCGGTGGCGACAACACCAACGCGAGCTGGACAGAAAGCGCGGTACGG	2450	2701	GTCTCGCATGGCATGGTTCGATCGCGCGCTGGCGGTCCGATGGTCCATGCC -----+-----+-----+-----+-----+ CAGACGCTACCGTACCACTACGAGCTACGGCGGCGCCAGCTCTACCAAGGTACG	2750
				V C D A M V D A A C A V E M V H A	
2451	CCTGACCGCATGTGGCAGCGCGCATGGGCGTTTGGCGATCGCGTCCGATGA -----+-----+-----+-----+-----+ GCACTCGCGCTACACCGTCCGCGTACCGCGAAGCGGTAGCGCCAGCGTACT	2500	2751	CGCATCGCTGATCTTCGACGACATGCCCTGCATGGACGATGCCACGACCC -----+-----+-----+-----+-----+ CGGTAGCGCATAGAAAGCTCTCTACGGGACGTACCTCTCTACGCTCCTGGG	2800
				A S L I F D D M P C M D D A R T R	
2501	CTGACGCAAGGACACCATGACGCCAAGCAGCAATTCGCCCTACCG -----+-----+-----+-----+-----+ CACTCGGTTGCTTCGCTGCTACTGCGGTTGCTGTTAAGGGCGATCGG	2550	2801	GTGCGCTCAGCGCGCGCACCCATGTCCGCCATGGCGAGGGGCGCGGCTG -----+-----+-----+-----+-----+ CAGCGCCAGTCGGGCGGTGGGTACACAGGGTACCGCTCCCGCGCGGCCAC	2850
	crtE --> M T P K Q Q F P L R			R G Q P A T H V A H G E G R A V	
2551	GATCTGGTCGATCAGGCTGGCGCAGATCTCGGCGCAGTTTCGGCGTGGT -----+-----+-----+-----+-----+ CTAGACCACTCTAGTCGGACCGGCTCTAGAGCGCGGTCAGCGCGCAACA	2600	2851	CTTTCGGCATCGCGCTGATCAGCGAGGCCATCGCGGATTTTCGGCGAGGC -----+-----+-----+-----+-----+ GAAAGCGCGTAGCGGCGGCTAGTGGCTCGGTACGCGCTTAAACCGCGCTCGG	2900
	D L V E I R L A Q I S G Q F G V V			L A G I A L I T E A M R I L G E A	
2601	CTCGGCGCGCTCGGGCGGCGCATGACGGATGGCGCGCTGTCCCGCGCA -----+-----+-----+-----+-----+ GAGCGGGCGGAGCGCGCGGCTACTGCTACGGCGGCGACAGGGGCGCGT	2650	2901	GGCGCGCGCAGCGCGGATCAGCGCGCAGGGCTGGTGGCATCCATGTGCG -----+-----+-----+-----+-----+ CGCGCGCGCGCTCGCGCGCTAGTGGCGGTTTCGACCGAGGTAGGTACAGCG	2950
	S A P L G A A M S D A A L S P G K			R G A T P D Q R A R L V A S M S R	
2651	AACGTTTCGCGCGCTGCTGATGCTGATGGTGGCGCAAGCTCGGCGCGG -----+-----+-----+-----+-----+ TTGCGAAGCGCGCGCAGCACTACGATACGAGCGGCTTTCGAGCGCGCGC	2700	2951	GGCGATGGGACCGGTGGGCTGTCCGCGAGGGCAGGATCTCGACCTGCAC -----+-----+-----+-----+-----+ CGCGCTACCTGGCGCGCGCGCACACGCGTCCCGTCTAGACCTGCACCGTGG	3000
	R F R A V L M L M V A E S S G G			A M G P V G L C A G Q D L D L H	

Fig. 7/6

3001	G C C C C A A G G A C G C C G C C G G A T C G A A C G T G A C A G A C C C T C A A G A C C G G + + + + + C G G G G T T C C T C G G G G G C C T A G C T T G C A C T T G C T C G A G T T C T G G C C + + + + + A P K D A A G I E R E Q D L K T G	3050	3301	A G C C C G C G C A A C T C G A C G A C G T G A T C G G C A C C G C G C T G T T C C G C G G G G + + + + + T C G G G C G C G C T T G A C C T C T C A C T A C G C G T G G C C G A C A A G C G C G C C C C + + + + + S R A Q L D E L M R T R L F R G G	3350
3051	C G T C T C T T C G T C G C G G C C T C G A G A T G C T G T C C A T T A T T A A G G C T C T G G + + + + + G C A G A A C G A G C C C C G G A G C T C T A C G A C A G G T A A T A T T C C A G A C C + + + + + V L F V A G L E M L S I I K G L D	3100	3351	G C A T C G C G A C C T C T G T G C C G C C G C G C G C T G C G C G C A T C A C A T C C G C G C A + + + + + C G T C T A C G C C C T C A C A C A C C G G G G C A C G A C G G G T A C T G T A G C G C G C G T + + + + + Q I A D L L A R V L P H D I R R S	3400
3101	A C A G C G G A G A C G A C C A C C T C A T G C C C T T C G G G C T C A C C T T G C T C G G + + + + + T G T T C G G C T C T G C T C G T C G A G T A C C G G A G C C C G C A G T C G A A C C A G C C + + + + + K A E T E Q L M A F G R Q L G R	3150	3401	G G C C T A G C G C G C G C T C G G G T C C A C A G C C C G T C C G C G C T G A T T T C G C G + + + + + C G C G A T C C G C C G C C A C C C A G G T T C C G G C A C C G C G A C C G C G A C T A A A G C G G C + + + + + A * A R P R T W L G D R S I E G	3450
3151	G T C T T C C A G T C C T A T G A C G A C C T C T G C A C C T G A T C G G C G A C A G C G C A G + + + + + C A G A A G T C A G G A C T C T C T G A C G A C C T C C A C T A C C G C T G T T C C G G T C + + + + + V F Q S Y D D L L D V I G D K A S	3200	3451	C C G G A G C C G C G A T G C G G C C G C G C G T C C A A G C T C C G C G C G C A A G A G C C + + + + + G C C C C T C C G C C T A C G C C G G G C A G G T T C G G A G C C G C G C G C G C T T C G G G + + + + + G R L R S A A A D L G G R A L L G	3500
3201	C A C C G C A G G A T A C G C G C G G A C A C C C C G C C C G C G C C A A G G C G C + + + + + G T C C C G T T C C T A T G C C G C C G C T G T G C G C G C G G G C C G G G T T T C C G C + + + + + T G K D T A R D T A A P G P K G G	3250	3501	G A T C T T G C A G C C T T C A A C G T C C A T C C G C T G C G A T A G C C C T C G C G C + + + + + C T A A A C C C T C G G A A G C T C A C G A C T A G C G A C C G C T A T C C G A G C C C G + + + + + I K A A K S T S I R Q R Y A E P	3550
3251	G C T G A T G C G C G T C G A C A G A T G C G C A C G T G C C G C A G C A T T A C C G C C + + + + + C G A C T A C C C C A C C C T G T C T A C C G C T C C A C C G C T C G A T G C T A T G C T C G C G C + + + + + L M A V G Q M G D V A Q H Y R A	3300	3551	C A C C T C C C G A T C C C G C G T C C G A T T C C G C A T A G A T A C C A G C G C G C G + + + + + G T G G A C G C C T A C C G C A C G C C T A A C G C C T A T C T A T G C T C G C G C G C + + + + + G G Q R I R T G I A R Y I R L A A	3600

3DOCID: <EP__0747483A2_1.>

3601	CGCATGACCAAGCGGCGCGGCGGAGATGCGGAGCGCGCTCGCGCGC -----+-----+-----+-----+-----+-----+ CGCTAGCTGCTCGCGCTCGCGCGCGCTCTACCGCTTCGCGGAGCGCGCG	3650	3901	CCACAGCCCCCGGAGCTGCTAGGAAATATTCAGGCACTGTCATCCAGCT -----+-----+-----+-----+-----+-----+ GGTCTGGGGCGCTCCACCATCTTATAGGTCGTGCAGTAGGTCGGA	3950
	A I S W A C R P P L H P L G Q R A			G V V G A V R Y S Y E L V D D L S	
3651	CGAGCATATAGCGCTCGCGCGCTCAAGCAGCGCGATGATGACGGAAT -----+-----+-----+-----+-----+-----+ GCTCGGTATATCCGAGCGCGCGCAGTTCTGCGGCTACTACTGCTTA	3700	3951	GGGTATTCCGATTCGCGGACATCCATCCGGAACCTCGATCGAGTCCCA -----+-----+-----+-----+-----+-----+ CCCATTAAGCGGTAGCGGCTGTAGTAGCGCTTTCGAGCTAGTCCAGGT	4000
	S A Y Y P E A A D L L R I I V S			R Y E R D A V D M A F G E I L D	
3701	AGCGCGTCCGAGCGACCGACCGCTCAACCGTCGCCCGCGCTCGGCC -----+-----+-----+-----+-----+-----+ TCTCGCGCAGCTTCGTCGCGCTCGCATGTTCCAGCGCGGCGGCGAGCGG	3750	4001	TGCGCAAGAGTCCGCGGAATCATGCGCGCGGCGACCTGGGCGCAGCGCC -----+-----+-----+-----+-----+-----+ AGCCGCTTTCAGAGCGCTTATGTTAGTACGGCGCGCGCTGACCGCGCTCGCGG	4050
	Y L A D S P V P G E V T A G A E A			M P W L D P F D H R R A V Q R L A	
3751	AGCAGTCCGAGCGAGATAGCAGCGCGCGATCGCGCATGCTGCATCAC -----+-----+-----+-----+-----+-----+ TCGTGACCGTCCGTCTATCTGTGCGGCGCTACCGCGGTAGCAGCTAGTG	3800	4051	CGGAAGCGCGCGACATTCGCGCGCTCTGCTGCAGCGCGGCGCAGCTGTC -----+-----+-----+-----+-----+-----+ CGCTTCCCGCGCTGTAGTACCGCGCGAGAGCACTGTCGCGCGCTCGCACAG	4100
	L W D A P L Y C R G I A A D D I V			A F P P S M P G D E H L A A L T D	
3801	GTCCGAGCGATGTTGTCAGCTCGAAGCGCGCGCGATCGCAGCGGC -----+-----+-----+-----+-----+-----+ CAGCGCTGCTACAGCAGTGCACCTTGGTTCCGGGCTAGCGTCCGGG	3850	4101	CGCGCGAGCGCGCGCGCGCGCTGTGCTGCGCGCGCGCTCGCGGCGG -----+-----+-----+-----+-----+-----+ CGCGCGCTCGCGCGGCTCGCGCGCGGACACCCAGCGCGCGCGCGCGCGCG	4150
	D R A I N T L Q F A L G L D C A			A R L A G L R A Q P D G G A E P	
3851	GATCCAGCAGCGCATGCTGCTGACGCCCATCAACCGCGCGCATCATCAG -----+-----+-----+-----+-----+-----+ CTAGGTGCTGCGTAGCAGGCGTGGGCTATGTGGCGCGGTAGTAGTC	3900	4151	CAGAACCATCATCTGCGCGCTGCATCATGTCATCCGATGCTGCACGAG -----+-----+-----+-----+-----+-----+ GTCTTGGGTAGTGAAGCGGACGCTAGTGCAGTAGCGCGTACGAGCGTGTG	4200
	R D L V A D D Q V G M V R A M M V			A S G M V Q G D I V D D A H R C W	

Fig. 7/8

4201	GCATAGACCATGACCTATCCTCGCGGATCGCGGGCGCATCAGCTTGGC +-----+ CGTATCTCGTACTGGCATAGAGCGCTACGCGCCCGCGTACTCGAACCG +-----+	4250	CCTGATGGCGCGACAGTTTCGGTGTGAATTCGGCGGGCTGAACATCGGG +-----+ GCATACCGCGCTGTCAAGCACAGACTTTAGCGCGCCCGGACTTCTACGCC +-----+	4501	G R H A S L E T S F D A P S F I R +-----+	4550
4251	A Y L M V T D E R I G P P M L K A +-----+ CGCTGCGCGAGCTTTCCGACCCCTGGCGATGGCGGCTTCGGAGTGG +-----+ GGGACCGCGCTTCGAAACGCTTGGGACCGCTACCGGCGAGCGCTTCAGC +-----+	4300	CTGACGTCAGTGTCTTCGCGAGTCCGGATGGCGCGCGCTCCAGTTTC +-----+ GACTGCCAGTCCACGAAACGCGTCAGCGCTACCGCGCCCGCGAGCTCAAG +-----+	4551	S V T L H K R L D P I A R R E L E +-----+	4600
4301	A Q A F S Q S G Q A I A A E S T +-----+ CGCTCAGATCGGTATCGGACGGCGAGGTCCGACAGCATGACCTGGCGCG +-----+ GGAGTCTAGCCAGTACGCTGGCGTCCAGCGCTGTGTACTCGACCGCGC +-----+	4350	CTCAGAGATCGGCTCGCATAGCCGGGCGCTCGGCTTCCCATCGACAT +-----+ GAGCTTCTACCGAGCGGTATCGCGCCCGGAGCGCGAAGGCTTAGCTGA +-----+	4601	E F I R E A Y G P A E A E W D V +-----+	4650
4351	TGGCTTGGCGCTGCCAAGCACCCCGGATGCCCGCACCGGATCGGTG +-----+ ACCGAACCAGCAGCTTGTGTGGCGCTAAGCGGTGGCGCTAGCGCAC +-----+	4400	CGCGCGCGCGATCGGAAAGCGCGCGAGCAGTAATCGTGGACATC +-----+ GCGCGCGGCTCTACGCTTTCGCGCGTTCCTGCCATTAGCGACCTGTAG +-----+	4651	D A R G L H P V P A L V Y H T S M +-----+	4700
4401	CGCGCCCGACGATGTAGAGTTCCGGATCGCGGCTCGCGTTATCGCG +-----+ GGCGGGGTGCTACATCTTCAGCGCTAGCGCGCGCGCGCCCATACCGC +-----+	4450	CGCTCGCGGCTCAAGCTCGCATTCGTCACCGAGGGCGCAATCCACATACAT +-----+ GGAGCGCCCGCTCCGACCTAGCGAGTGGTCCGCTTACGCTCTATGTA +-----+	4701	G E P A L S P D T V C P S H L Y M +-----+	4750
4451	GGGACCAAGCGGATTCGCTCAGATCGGCTCGACCGAGAGCGCGCTGC +-----+ CGCTTGGTCCGCTAACCGAGTCTTAGCGGAGCTGGCTCTTCGCGAGC +-----+	4500	CGAATAATGCTCCGCGACGCGTGGCGCGCTTGAAGATCTCTCTCACAGCC +-----+ GCTCTTAGCAGGCGCTCCGACACGCGCGAATCTTAGAGCAAGTGGTGGG +-----+	4751	S F D D P L R P G N F I E N V L +-----+	4800

Fig. 7/9

4801	CCTTGTACCGCGCGCGAAGATGACGCTGTGCTGGCCAGCGTTCTCGGG -----+-----+-----+-----+-----+ GGACATCGCGCGCGCGCTTCTACTCGGACACACACCGCGTCCAAAGACCGCC G K Y R P G F I V S H H A L N E P	4850	5101	GCTCGAACAGGCGCGACCATGCGCGGACGACGCTGTTGTCGCGCGCTTG -----+-----+-----+-----+-----+ CGACGTTCTCGCGCTGTTACGGCGCGCTGTCGACCAACCGCGCGGAGAC R E F L A V M G A V L Q N T G G K	5150
4851	CGCTTGACAGCGCGAATGCAGCAGAACCGACATCGACAGCGCTG -----+-----+-----+-----+-----+ GGACACCTGTCGCGCTTACGTCGCTGCTTGTCCGCTGTAGCTGTCGGAC R K S L G F H L V F L S M S W R Q	4900	5151	GCGAACAGAGCGCGCGCGCGCTTCCAGCGCATGCTACAGCGCATAGAT -----+-----+-----+-----+-----+ CGCTTGTCTCGCGCGCGCGCGCGCGCGCTGCTACCTAGTCCGCTATCTA A F W V G G R R E L A H I L A Y I	5200
4901	CGGTTTCAGATCGCGCGCTTGTGCGCGCGCGCGCGGTATGCGCCAGCA -----+-----+-----+-----+-----+ GCCAAGTCTAGCGCGCGAAGACAGCGCGCGCGCGCGCATACCGGTCGT R N L I A A K T R G R R T H G L	4950	5201	CGAGCTGTCGAAACCGGTTCCGCGGACGACGCGCTGTGGAACGAGA -----+-----+-----+-----+-----+ GCTCGACAGCGTTTTCGCGACGGCGCGCTGTCGTCGACACCTTCTCT S S T S F P N G G V L L T H F S	5250
4951	GCTCGCATAGCTGTGCATCACTGTCGCGCTTGTGCGCACCGTATCCGCG -----+-----+-----+-----+-----+ CCAGCGCTATCGACACGTACTGCGAGCGCGACGACCGGTCGCTAGCGCGC L D R Y S H M V D G N S A V T D A	5000	5251	AGCGCTCGCGCAGATGCGGTCCTGCTGATGAAGCGCGCACCATGCTGTGG -----+-----+-----+-----+-----+ TCCGAGCGGCGTCTACGCGCAGGAGCTACTTCCGCGCGTGTGTCGACACC F A Q R L H P D Q I F R A V M S H	5300
5001	CGCAACTCCGCGCGCTGCACAGCGTCAACGCGCGTGGCGCGATCCGCGTC -----+-----+-----+-----+-----+ CGCTTCAAGCGCGCGCGAGTGTGTCGCACTGCGCGCACACCGCTAGCGGAG R L Q R G D L L T V G T A R D G E	5050	5301	ACCGACCGGTATGCTTCAGCGCGCATCAGCGCGCGCGCGCTTCAGCAT -----+-----+-----+-----+-----+ TGCTCCCATACGACGCTCCGCTAGTCCGCGCGCGCGCGCGCGCTGTA V S R Y A Q L R M L A P A A N L M	5350
5051	GCTGTGATCCGCGTCAAGCGCGCATTCAGCAGCAGCGTCCGCGCGACAC -----+-----+-----+-----+-----+ CCACAGCTAGCGCGCTCCGCGCGGTAGTGTGTCGTGCGACAGCGCGCTTCTG T D I R T V R A N L L L T G G L	5100	5351	CTGCGCGAGCTTCAGCAGCGCGTGTCCGCGCTTCAGATACCGCTGCG -----+-----+-----+-----+-----+ GACGCGTCGACAGTCTTCGCGCGCGCGCGCGCGCGCTGAGTCTATCGGAGCG Q G L K L F P T T G L K L Y G E	5400

Fig. 7/10

5401	GATAGACCTCTCGGGGTATTCGTGGAGCGCGGATAGCCATCGACATCG CTATCTGAGAGAGCGGATTAACACCTTCGGCGCTATCGGTAGCTGAGC R Y V E E A Y D H F R R Y G D V D	5450	GGCCCTCGACGATCGTGTGCGATCGCGCGGATTCGAGCGGATGGCA CCCGAGCTCTACACCGACGCGCTACGGCGGCTACGTCGGCTACCGT R A E V I T T A I G A S Q L R I A	5701	5750
5451	GGCGATTGAGGAGCGGACCTCGCGGATCAGCTGCTGCTGCTGCTTTCAC CGCCCTAATCTCTCGCTCGACCGGCTAGTCGAGCAGCAGCAGCAGGTG A P N F S A V Q R I L E D D D N V	5500	AGCGCAAGCGCGCGGAGAACCTTCGGCGGATGACGATCGCGCAACTCATCT TCGGTTGCGGCGGCTTTCGACGCGGCTACTGCTACCGGCTTCGAGTACGA L A L G G F G A G I V I A S S M <-- crtI * A	5751	5800
5501	GTATTGGAAGCTCGCGCGCTCGCGGATGTCAGCGGCTAGAGCGGCGAGA CATAGCTTCGACGCGCGGCGGCTACAGTCGGGCGATCTTCGCGGCTCT Y E F S R G D A W T L R Y F P S	5550	CTCTCTCGACGAGCGGCGGCTTCGGCGAGCGAGCGGACCGGCTCGGACAG GAGAGCACTGCTGCTCGCGCGGAGCGGCTGCTGCGGTGCGCGAGCGCTGTC R E Q L L P R E P L C R V A Q S L	5801	5850
5551	CGGAGCAGCGTCACTCAGCTCCTCATCTGCTGGGCGGCTGAGCGGCGCAC GGCGTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT V P L L T V D R E M P Q G S L A W	5600	CGGATGCGCGGCGGCTCGGCTGAGATCGGAGCGGCGGCTCGGCGGATGTCA GCTTACCGCGCGGCGGAGCGGCTGCTACGCTTCGGCGAGCGGCTTACAGT P I P P R G T V I R L R D A L T	5851	5900
5601	AGCTTCGCGAGCTTCGGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT TCGAGAGGTCGAGACGCGCGGCGGCTGCTGCTGCTGCTGCTGCTGCTGCT L E R L S D P D T V V T P G A D F	5650	GGCGCGCGCATAGAGCGGCTCGATCAGCGGCTCGGCGAGCGGCTAGAAC CGCGCGGCGGCTATCTTCGCGAGCTGCTGCGCGAGCGGCTCGGCGATCTTG L R G A Y F R E I L P Q P L R Y F	5901	5950
5651	CAGCTGCGGCTGATGCTTCGACAGCATAGCGCGGCGGCGGCTTGTGCGC CTCAGCGGCTAGCAGGCTGCTGATCGCGCGGCGGCGGCGGCGGCGGCGG V H G Q D N W V Y A R G G P K D	5700	CGCTGCGAGCGGCTAGCGGATCGGTCGGCGGCGGCGGCGGCGGAGCAGCAT GGAGCTGCTCGGCTATCGCTGCGAGCGGCGGCGGCTCGGCGGCTTGTGCTA R Q L L R Y R R D P P C G R F L M	5951	6000

Fig. 7/11

6001	CGCGTTACGCAACCGGCAAGAAAGCGGTGCGCATTCGCGCGCATTCGATGCGCC	6050	6301	CAGCGACGCTGCGCCATGCGCGCCATTCGTCGAGATCGCGCGCCGCTGCGTGT	6350
	CGCCAAAGTCGTGCGCGTCTTCCGCAAGCGCTAGCGCGGCTAGCTAACCGGG			GTGCGTCGCGAAGCGCGTCTGCGCGGCTAGCGAGGCTTACGCGCGCGCGAGACA	
	R N L L P L F R D R D A R D I A			L S A Q A L A G D D L D G G D S	
6051	AGCGCGCAACCGCGGAGCGCGCGGACGCGGTGTCAGGTGCGCGCGCGCGG	6100	6351	AGCGGTATGCTCTGATCAGAGATGCGCGTGGGACTGAAAGCGCGACAGATAG	6400
	TGCGCGCTGCGCGCGTGGCGCGCTGCGCGCGCTGCGCGAGCGAGTCCAGCGCGCGCGG			TGCGCGATAGGAGCTACTCTTACCGCGACCGCTGACTTCGCGTCTGCTATC	
	W G R V A R R A S A T T L D R A A			Y R T D E I L I R T P S F P L L Y	
6101	ATGCAATCGCGACCTGCGCGCGGATAGCGCAAGCAATATCGCGTCAACGGG	6150	6401	ATGAAAGCGGTACCGGTCCATCTCGCGAAGCGTCCGCTCCATGATCATCGG	6450
	TACGTAAGCGCTGACAGCGCGGTATCCCGTCCCTTATAGCGCACTGCGC			TACTTCCCATGGGCAAGTAGACCGCTTGGCGAGCGGAGCTACTAGTAGCC	
	I A D A V Q A A Y P L S Y G T V P			I F R Y G D M Q P V T A D M I M P	
6151	GTGCAAGCGCTGCGCGCGAGCGCGCAAGCGCGCGCGCTGCGGTGCT	6200	6451	GCGCTCAACCGCATGCGCGCGGTGCGTCTCGATCTCGAAGCGCGCAAGATT	6500
	CACCTTGTGCGAGCGCGGTGCGGTTGCGCGTGGCGGCGGAGCGCGCAACA			CAGCACTGCGGTACCGCGCGCGCAAGCGAGCTAGAGCTGCGCGGTGCTTAA	
	H F L G A G L G V P V A G Q A H			R E V G H P A D T E I E V G V F	
6201	CGCGCAAGCGCTATGCGGTCTATGCGCGCGAGCGCGATGCGCGAGATGCGC	6250	6501	TCTGGAAGCGGACGCTCAGGTGCGCGGTCTCGAAGCGCAAGCGCGGTGCG	6550
	CGCGGTCTTGGGATACCGCATACCGGTGCGCGTACCGGTACCGG			AGACCTTTGCGTCCCATGTCATCGCGCGAGAGCTGCGGTGCTGCCCGAGC	
	D R W F G I A D H A L A I P L I G			K Q F G V T L H P T E V A G R A D	
6251	CTTTCGCGCGCATTCCTGCGCGGTCCAGCGCGCGCTGCGCGCATTACTC	6300	6551	ATCAAGCAAGCAAGCTCTCATTCGCGAGCGGTCCGTCAAGCTGCGCGCGGT	6600
	GAAGCGCGCGCTAGAGGAGCGCGCGAGGTGCGCGCGAGCGCGGTATCAG			TAGTGGTTCGTGCGAGCTAGCGCGCTGCGCGAGCTGCGAGCGCGCGCA	
	R E R R M E Q G T W G R R A A Y D			I V C A A E I R S G D T L T A G T	

Fig. 7/12

6601	ATGTCGAGGCTCGGACATCGGTATTCACGCGGATCGACACCTGCA TACGAGTTCGAGGCTGTACCATAAAGTGGGCTGTAGCTGTGGAGCT	6650	6901	6950	ACGGACAGCCCGCGCGCGCGGATCGACGATCATGGCTCATGTATGG TGCCCTGTGCGGCGCGCGCGCTAGTGTCTAGTACCGAGTACATAACG
	D D L T A V H T N W R L D V G Q				G S L G A G A I L L D H S M ←-- c r t y
6651	GCAGCCGATCAGCGCGCGCGCTCGATCGAGCCATAGCTGTGCTCAGG CGTGGGCTAGTCCGCGCGCGGAGCTAGCTCGGTATCGGACACGAGTCC	6700	6951	7000	ATCCGCCCTTCGGGCTCCTTCAGCGAGCGCGCGAGCGTTTCAGCTCTG TAGCGGGGAGCCAGGAGCTGTGCGCGCGGCTCGCAAGTCCAGAC
	L L G I L A G A E I S G Y G T T L				D A G E R D K L L A G S R K L E
6701	CGCGCGGATGTCGGGAAACCGGACCTCTCATCTCCATTCGCGCG GCGCGCTTACGACCTTTGCGCTCGAGGACTAGCGAGGTAAAGCGCGC	6750	7001	7050	CCTTGAGCTGTGACCGGCGCGCGCGGATGAAACCGAGCTGACCGAG GGACTCGGAGGCTGCTGCGCGCGGCTCTCTTTGCTTCGACTGCTGTC
	R R S H D P F A V E Q D T W E G R				A K L S D V S P A W I F G F S V C
6751	ACGATGCGGACAGCGCGCGGATTCGCGGAGGATCGCTGTGCT TGTACCGCTGTGCGCGCGCTCGGTAGCGCGCTTCTAGGACACGA	6800	7051	7100	TTCTCGCGGATCGAGCGCGCTCATCTCTGCTGCTGCTGCTAGACCGG AAGAGCGCGGTAAGTGGCGGCTAGCTAGGACACAGGACCACTGCGC
	R I P S L R A L W E P S L D T D				N E R G H V A H H M R H A Q Y V R
6801	CGCAGGACGAGCTGTGCTCGGAGGCGCGGACCGCGCTCGAGCATC CGCTCTGTGTCACAGGACGAGCTCGCGCGCGCTCGCGCGGCTGTAG	6850	7101	7150	ACGAGATACGCGCGCTTGGGACATAGCGGAGCGCGCGCGCATGCA TGTCTTATCGCGCGGAGCGCTGTATCGCTTGGCGGTGCGGTAAGT
	H C S W T H Q D S P G S R A D L M				R L Y O R K P V Y R F P W R G H
6851	ACGATCGCGGATCGGCTCGGTCGCGGAGCGGAGCGCGATCGAGCGC TGCTACGCGGATAGCGGAGCGGAGCGGCTTGGCGTTCGCGCTAGTCCG	6900	7151	7200	CCAGCGGCTCATCGAGGAGATAGTATGAGCGCGGATCGAGGTCACCGCC GTTTCGCGATGAGTCTCTTTATCATCTAGTTCGCGGATGCTGTCACCTGGG V L G D H L F Y Y I L G Y C T V G

[illegible]

Fig. 7/14

7801	CCCGGATCGGCATCGGCTGACCGGGCTTCAGGCGAAGGCGATCCGGCTC GGGCTAGCGGTAGCGGACTGGCCCGAAGTCGGTTCCGCTAGCGCGAG	7850	8101	GGGCGCTCTTCGGGGCTGTCCGCACTCGACCGGAACCGGAGCGTTTC CGCGGAGAGCCCGCAGCAGCGGCTGGAGCTTGGGCTTGGGCTCGCAGAG	8150
	G P H A M P Q G P K L G L R D A E			A R E E P S D A V E V R F G L T E	
7851	TCCGCCCGGATTCGAGGACGACAGCCGCTCGGGTCGCGATCGCGA AGCGCGCGCTAAGCTCTGCTTGTGGCGAGCCCGGCTAGCGGCT	7900	8151	CGCACCGGTATCGACGACAGACGCTCCGGGGCGGCTTCGACCGCGCGG GGTGGCCATAGCTGCTGTCTGACGGCGCGCGGCTAGGCTGGCGCGCG	8200
	G G A I E L V F L R D P D P D G			A G T D V V L S G P A C E V A A	
7901	CCGCCCGCCCGGATCGGCTCTGTCAGCGGGCGCGGATTCGGGTGG GGCGCGCGGCGCTTACCGCGACAGAGGTCCCGCGCGGTAAAGCGACG	7950	8201	CGCGCGCGCGGATCGAGACCGGAGAAAGGCTCGGGGCTTACTCGGCGAC GGCGCGCGGCTTACTGCTCGGCTTCTCGGAGCGCGGCTTACGCGGCTG	8250
	V A A G P I P T E D L P R A N R H			A A A P M L V A L L A A A K S P W	
7951	ATGTGGGGATGACGCGGTTTCATCCGCAAGAGCATCTCCAGCGGAT TACACCGCTACTCGCGGCGAAGTAGCGGTTCTGGTACAGGTCCGCTA	8000	8251	ATGGCGAAGTAGGCTCTCTCGGCGCGGAGATCTCTGACGCTCGGCT TACCGGTTCTATCTCTGACGAGCGCGGCTCTAGGACGACCTGGGACGCTA	8300
	I H R I V G T E D A F V M D L P I			M P L I P S S P A S I R S V R R M	
8001	CAGTGTTTGGCATCCAGAACGACACCGGCTGGCGGATTCGTAGATGA GTCACAGAACCGGTAGGCTTCTGTGGCGAGCCCGGCTAAGCATCTACT	8050	8301	CCTGTTTCCGCTCATCCAGCGCGGCTCCATCCGCGGATCTCGCGGAGC GACGAGGCGGATGATCTCGGCTTCAGGGTACGGGCTAGACCGCGGCGAG	8350
	L T N R M W F S V P Q P S E Y I			R T G T M <-- orf-16	
8051	ACAGCATTCGGTGGCGGAGGAGCTCTTTCGGAAATCAGGCGGCTGC TGTCTTAGGCGCAAGCGGCTCGCTCGAGGAAAGCGCTTGTAGTCGGGACG	8100	8351	ATCAGCGCGCGGAGCCCTCGAGCAGCGGAGGCGGATCCGCTCGCGGAT TAGTCGGCGCGGCTCGGAGCTCTCTCGGCTTCCGCTTCTAGCGGAGGCTA F L M G T G A P L E K R F M L G Q	8400

Fig. 7/15

8401	CACGAGGTCGAGAGCCCGAATGACGAGCACCTCGATATCGATGAACA -----+-----+-----+-----+-----+ GTGCTCCAGGCTCTTCGGGCTTACTCTGCTCGTGGAGCTATACCTACTGT	8450
8451	CGTCTCGGGTGCACGAGATGTTGGCGAACCGGGGAAGGCCCTTGGC -----+-----+-----+-----+-----+ GCAGGAGCCGCCACGGGCTTCTACAAACCGCTTGGGCTTTTCGGGGAAACGG	8500
8501	CTTGTGGAACCACTTGACGGGGGGCGGACCGCAGCGGCAAnnCGTCCAGATG -----+-----+-----+-----+-----+ GAACAGCTTGGTGAACTGCGCCCGGCTCGGTGCGCGTnnGCAAGGTCTAC	8550
8551	CTCGATCACCTCGGCATCCAGATCGGCGATnGGGGGGTnGnGTGCGCTTT -----+-----+-----+-----+-----+ GAGCTAGTGGAGCCGTAGGTCTAGCGGCTAnCCCCCAAGnGnGACGGAA	8600
8601	CnnnCGGTTGATCGACAGGACCTC -----+-----+-----+-----+-----+ GnnnGCCAAGCTAGCTGCTCTGGAG	8625

Fig. 8

1 MTPKQOFPLR DLVEIRLAQI SGQFGVVSAP LGAAMSDAAL SPGKRFR AVL
51 MLMVAESSGG VCDAMVDAAC AVEMVHAASL IFDDMPCMDD ARTRRGQPAT
101 HVAHGEGRAV LAGIALITEA MRILGEARGA TPDQRARLVA SMSRAMGPVG
151 LCAGQDLDLH APKDAAGIER EQDLKTGVLF VAGLEMLSII KGLDKAETEQ
201 LMAFGRQLGR VFQSYDDLDD VIGDKASTGK DTARDTAAPG PKGGLMAVGQ
251 MGDVAQHYRA SRAQLDELMR TRLFRGGQIA DLLARVLP HD IRRSA

Fig. 9

```

1  MTDLTATSEA AIAQGSQSFA QAAKLMPPGI REDTVMLYAW CRHADDVIDG
51 QVMGSAPEAG GDPQARLGAL RADTLAALHE DGPMSPFFAA LRQVARRHDF
101 PDLWPMDLIE GFAMDVADRE YRSLDDVLEY SYHVAGVVG VMMARVMGVQD
151 DAVLDRACDL GLAFQLTNIA RDVIDDAAIG RCYLPADWLA EAGATVEGPV
201 PSDALYSVII RLLDAAEPYY ASARQGLPHL PPRCAWSIAA ALRIYRAIGT
251 RIRQGGPEAY RQRISTSKAA KIGLLARGGL DAAASRLRGG EISRDGLWTR
301 PRA

```

Fig. 10

1 MSSAIVIGAG FGGLALAIRL QSAGIATTIV EARDKPGGRA YVWNDQGHVF
51 DAGPTVVTD P DSLRELWALS GQPMERDVTL LPVSPFYRLT WADGRSFEYV
101 NDDDELIRQV ASFNPADVDG YRRFHDYAE VYREGYLKLG TTPFLKLGQM
151 LNAAPALMRL QAYRSVHSMV ARFIQDPHLR QAFSFHTLLV GGNPFSTSSI
201 YALIHALLERR GGVWFAKGGT NQLVAGMVAL FERLGGTLLL NARVTRIDTE
251 GDRATGV TLL DGRQLRADTV ASNGDVMHSY RDLLGHTRRG RTKAAILNRQ
301 RWSMSLFVLH FGLSKRPENL AHHSVIFGPR YKGLVNEIFN GPRLPDDFSM
351 YLHSPCV TDP SLAPEGMSTH YVLAPVPHLG RADVDWEAEA PGYAERIFEE
401 LERRAIPDLR KHLTVSRIFS PADFSTELSA HHGSAFSVEP ILTQSAWFRP
451 HNRDRAIPNF YIVGAGTHPG AGIPGVV GSA KATAQVMLSD LAVA

Fig. 11

1 MSHDLLIAGA GLSGALIALA VRDRRPDARI VMLDARSGPS DQHTWSCHDT
51 DLSPEWLARL SPIRRGEWTD QEVAFPDHSR RLTTGYGSIE AGALIGLLQG
101 VDLRWNTHVA TLDDTGATLT DGSRIEACV IDARGAVETP HLTVGFQKFV
151 GVEIETDAPH GVERPMIMDA TVPQMDGYRF IYLLPFSPTR ILIEDTRYSD
201 GGDLLDGALA QASLDYAARR GWTGQEMRRE RGILPIALAH DAIGFWRDHA
251 QGAVPVGLGA GLFHPVTGYS LPYAAQVADA IAARDLTAS ARRAVRGWAI
301 DRADRDRFLR LLNRMLFRGC PPDRRYRLLQ RFYRLPQPLI ERFYAGRLLTL
351 ADRLRIVTGR PPIPLSQAVR CLPERPLLQE RA

Fig. 12

1 MSTWAAILTV ILTVAAMELT AYSVHRWIMH GPLGWGWHKS HHDEDHDLAL
51 EKNDLYGVIF AVISIVLFAI GAMGSDLAWW LAVGVTCYGL IYYFLHDGLV
101 HGRWPFRYVP KRGYLRRVYQ AHRMHHAVHG RENCVSFGFI WAPSVDSLKA
151 ELKRSGALLK DREGADRNT

Fig. 13

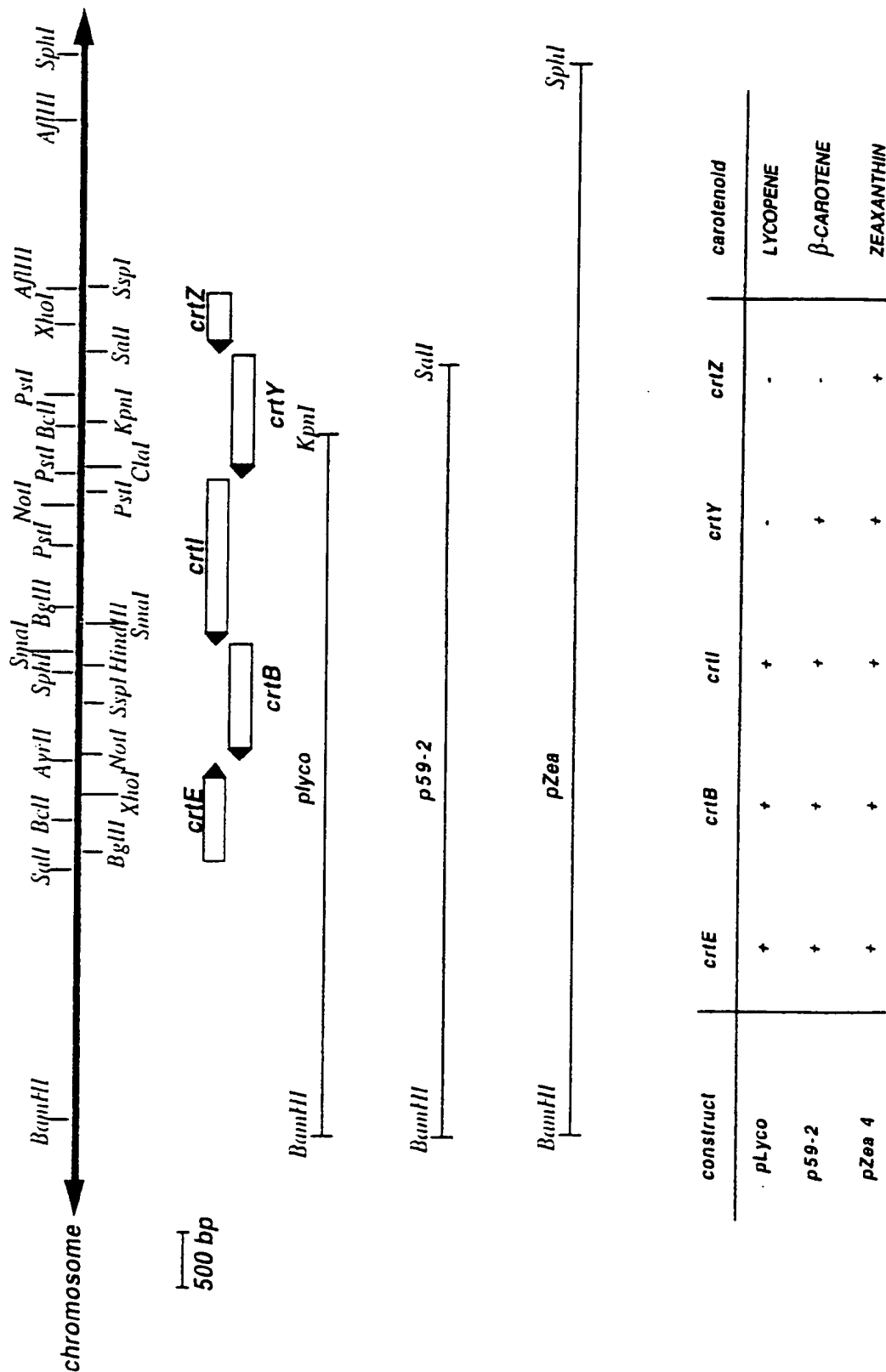
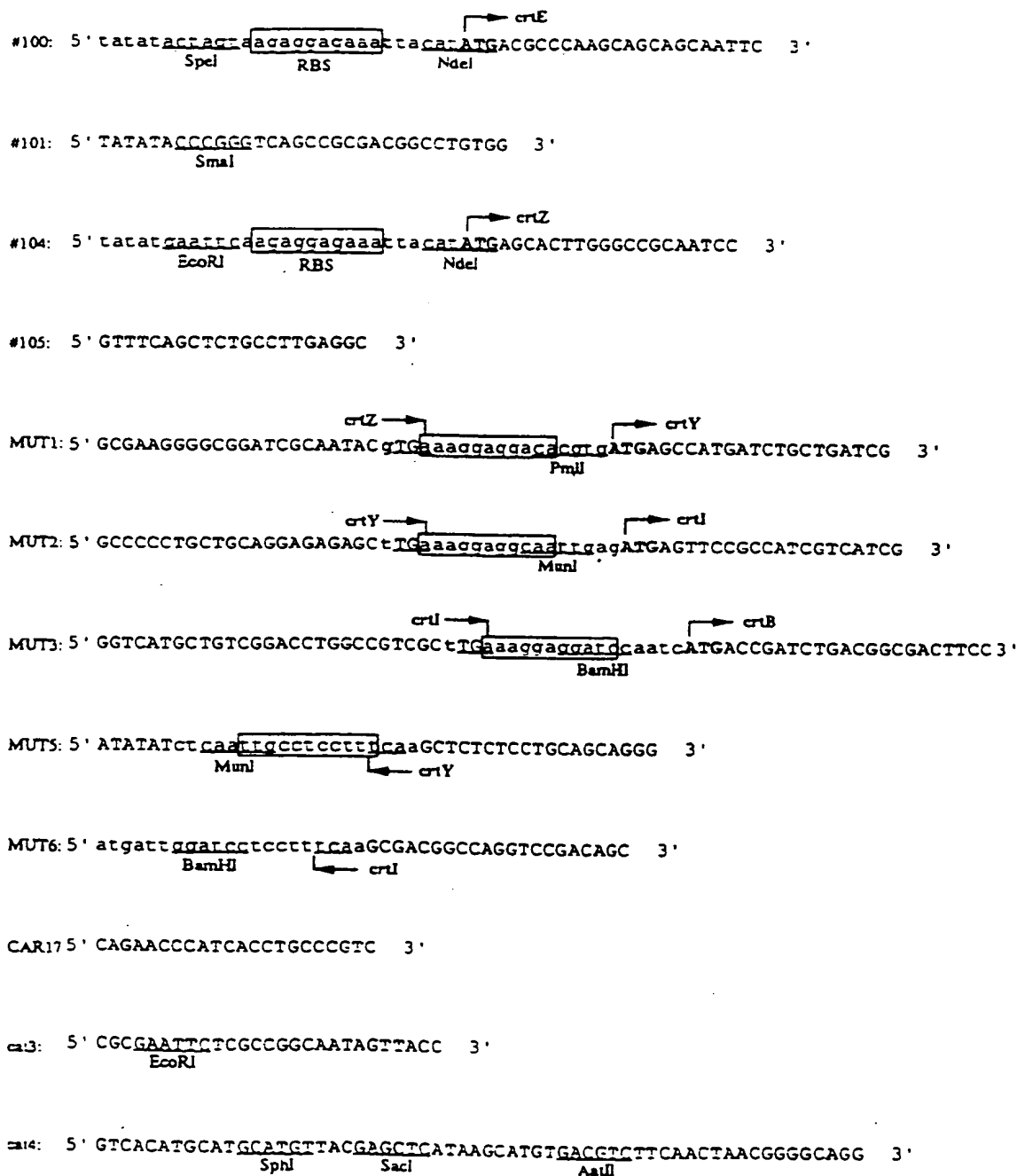


Fig. 14



HindIII AIII XbaI EcoRI
 CS1: 5' AGCTTGGATCCTTAAGTACTCTAGAGTTTAAACG 3'
 CS2: 3' ACCTAGGAATTTCATGAGATCTCAAATTTGCTTAA 5'
 Scal PmeI
 Sall AvrII MluI BamHI HindIII
 MUT7: 5' TCGACCCCTAGGCACGTTGACGCGTCAATTGGATCCGCATGCCAAGCTT 3'
 MUT8: 3' GGGATCCGTGCACTGCGCAGTTAACTAGGCGTACGTTCCGAACCTAG 5'
 PmlI MnlI SphI
 1/2 PmlI
 MUT9: 5' gtgtcctcctttcacgtatttgcgattccggcccttccggggtccttcagcagcggcccgagcggtttcagctctgcctttgaggctg 3'
 MUT10: 3' cacaggaggaaagtgcataacgcctagggcgagggaagcgcaggaaagtcgtcggcggggctcgcaaaagtcgagacggaaactccgacagct 5'
 RBS ← criz Sall
 Spel
 MUT11: 5' TAAGAAACcctcctttA 3'
 MUT12: 3' TCTTTGggaggaaaTGATC 5'
 RBS

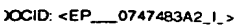
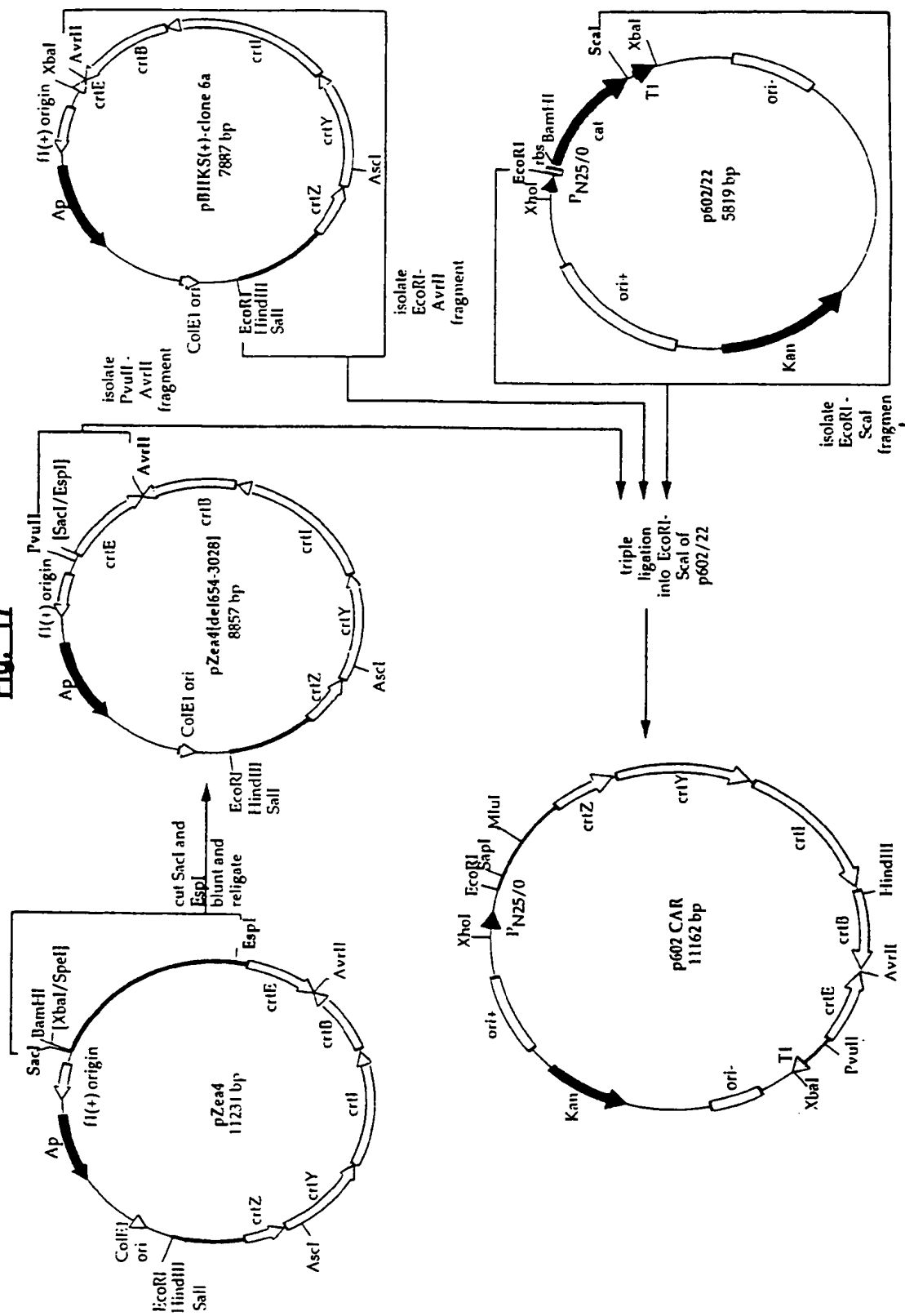


Fig. 17

[illegible]

Flg. 19

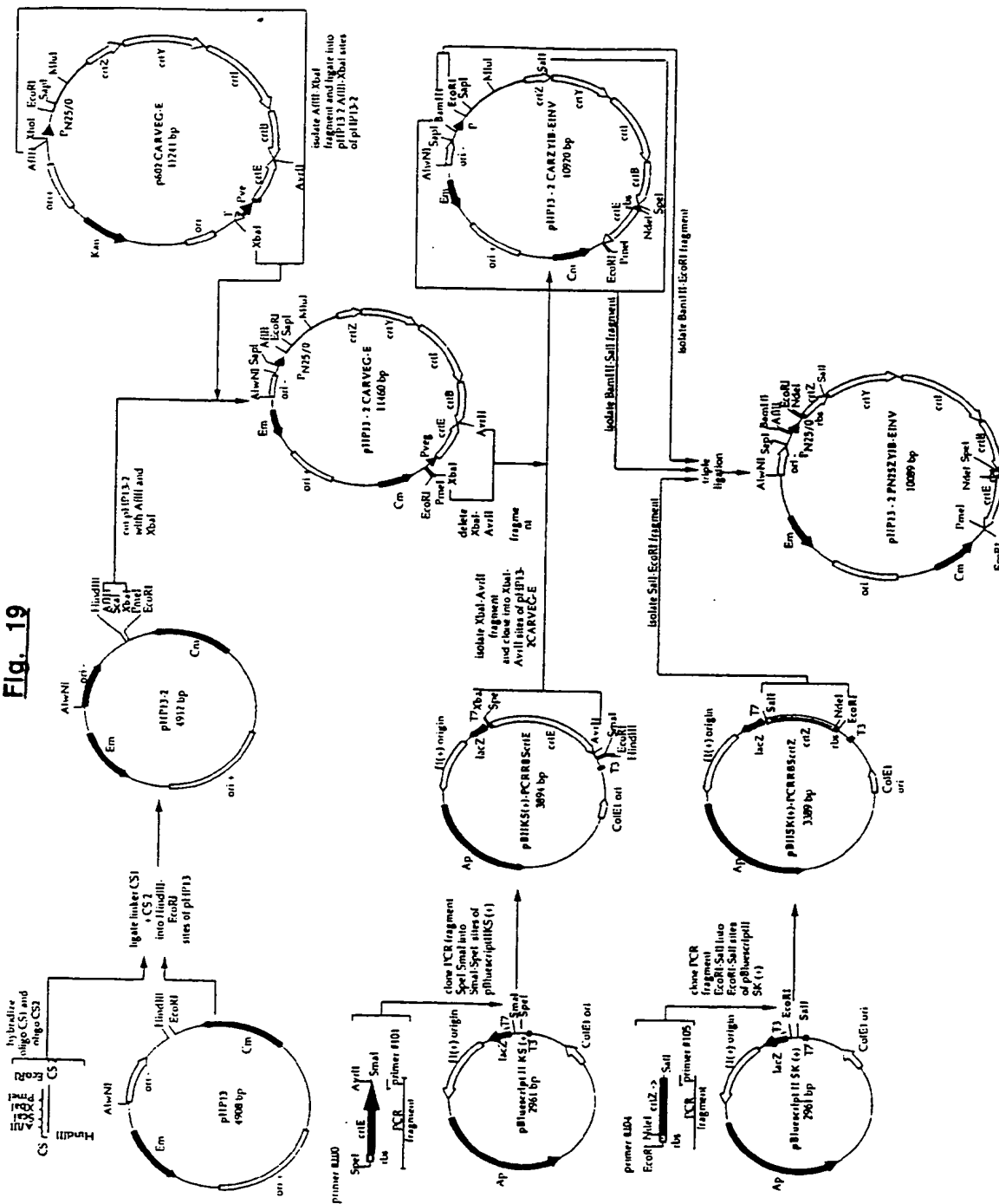


Fig. 20/1

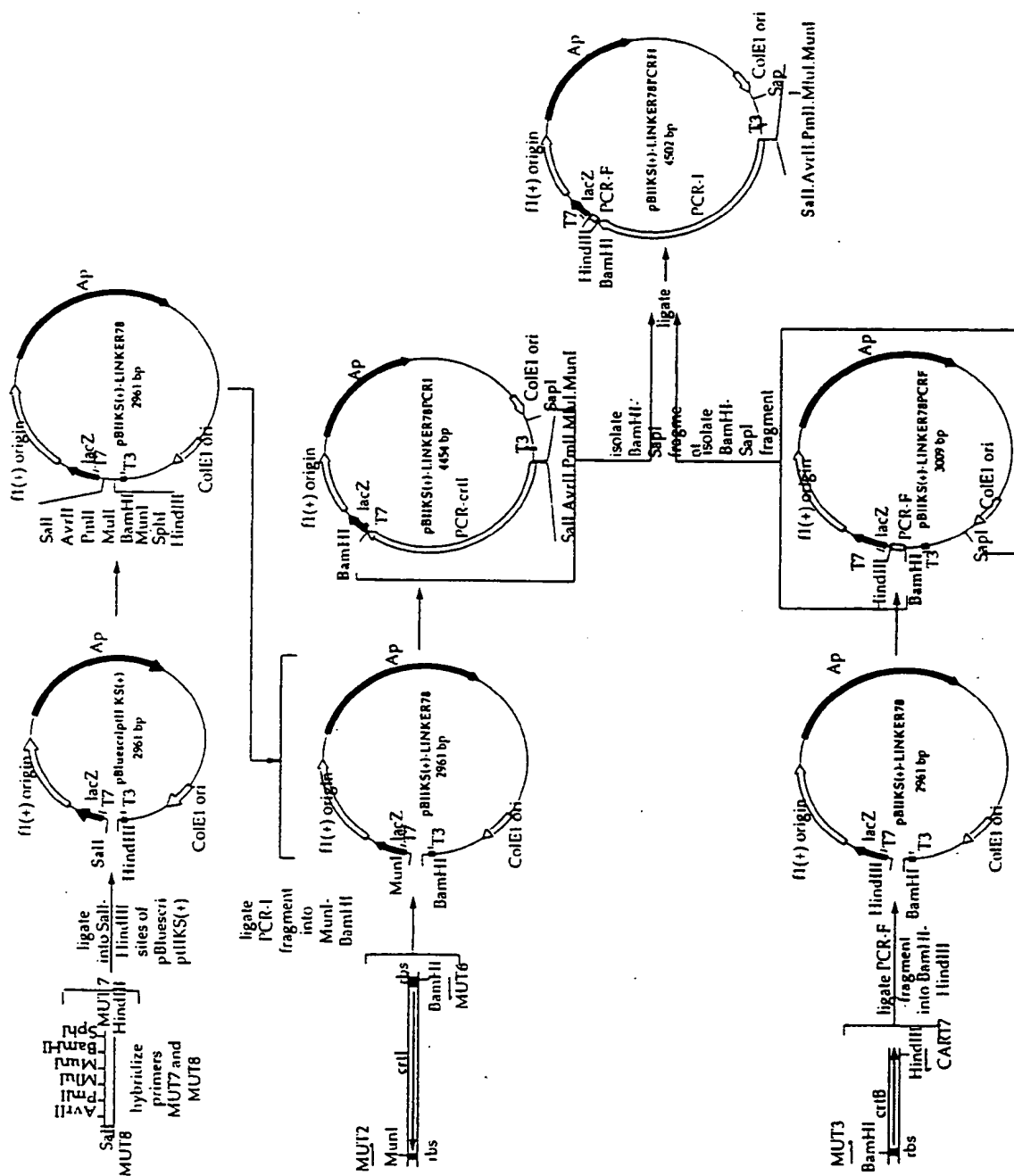


Fig. 20/2

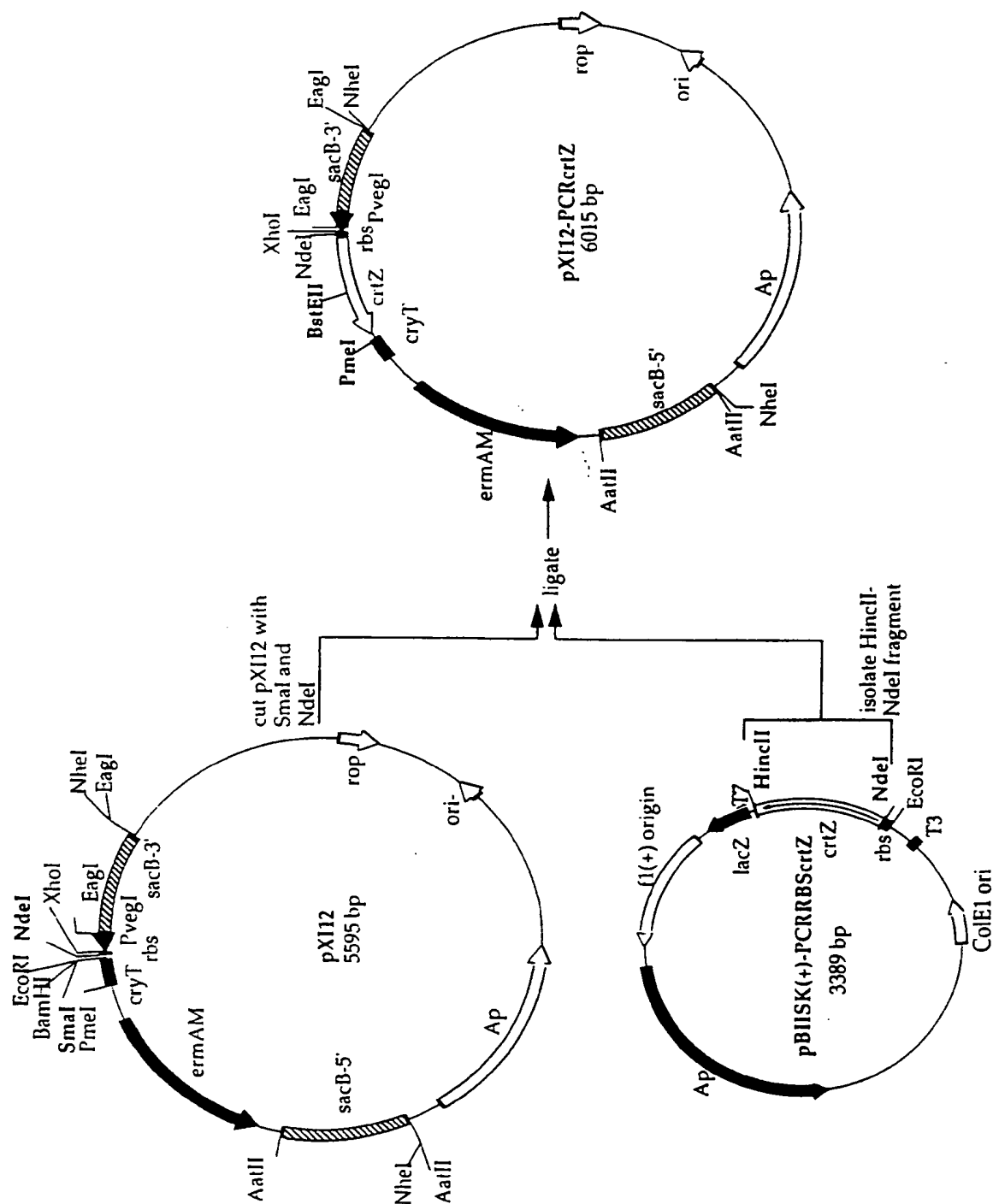




Fig. 20/4

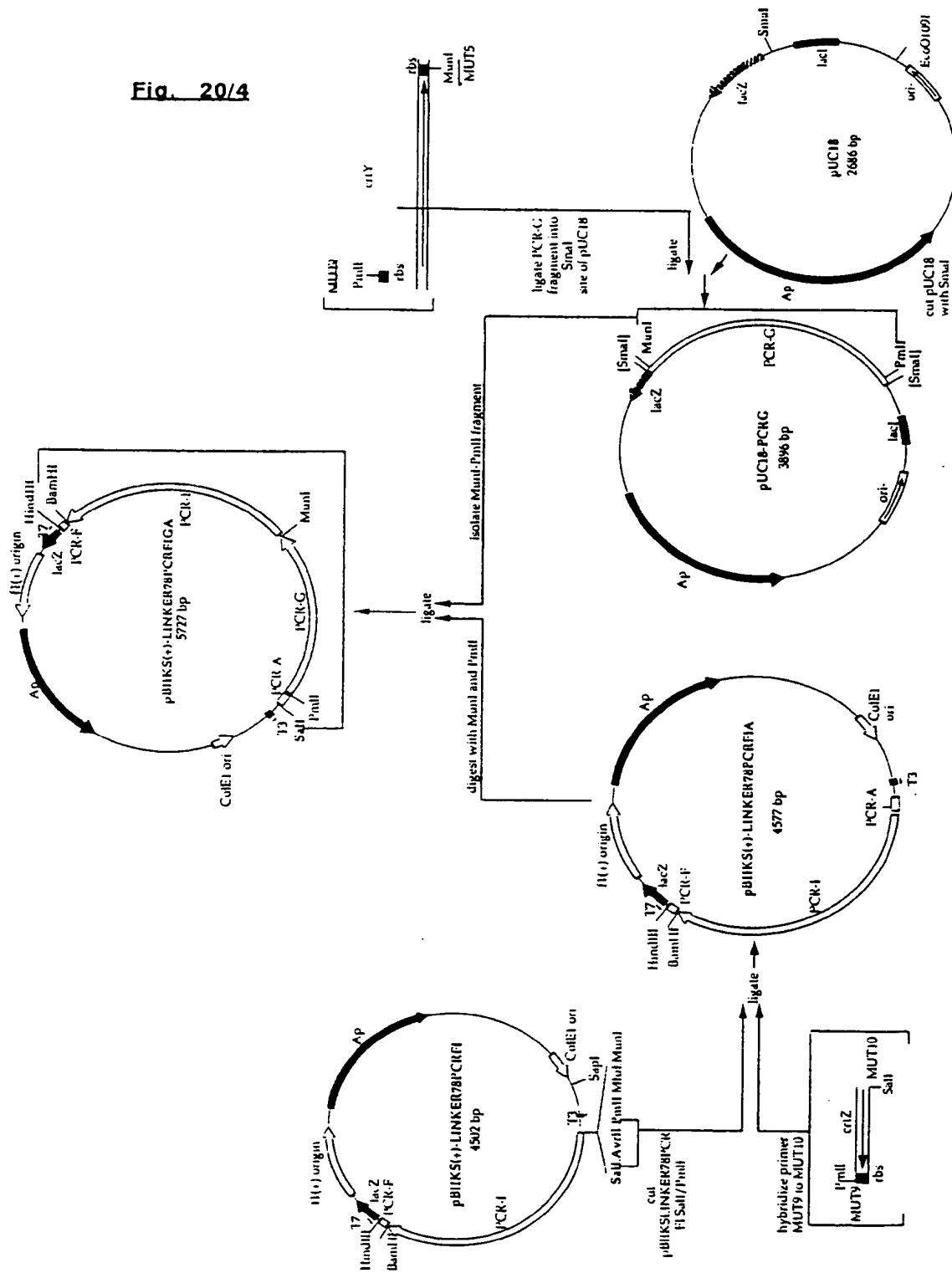


Fig. 21/1

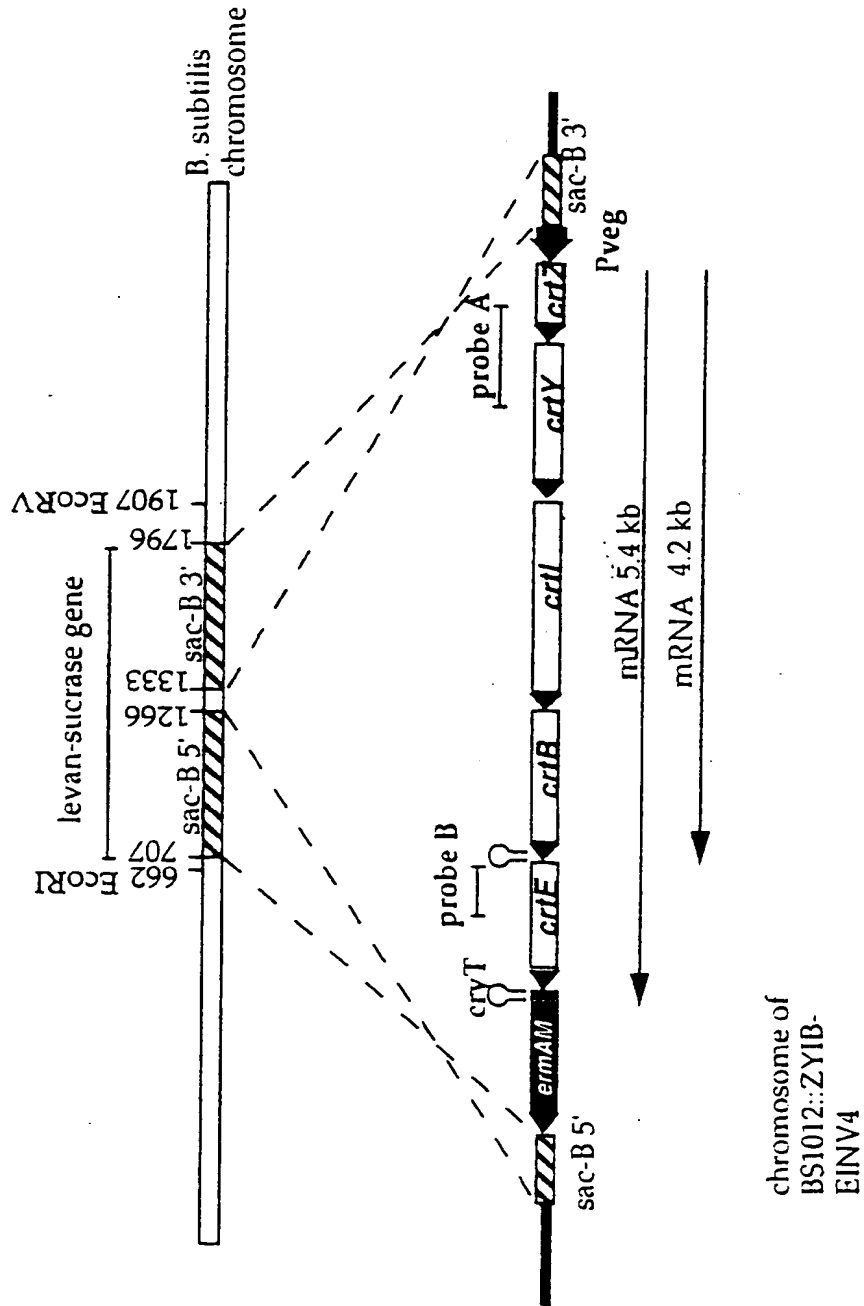


Fig. 21/2

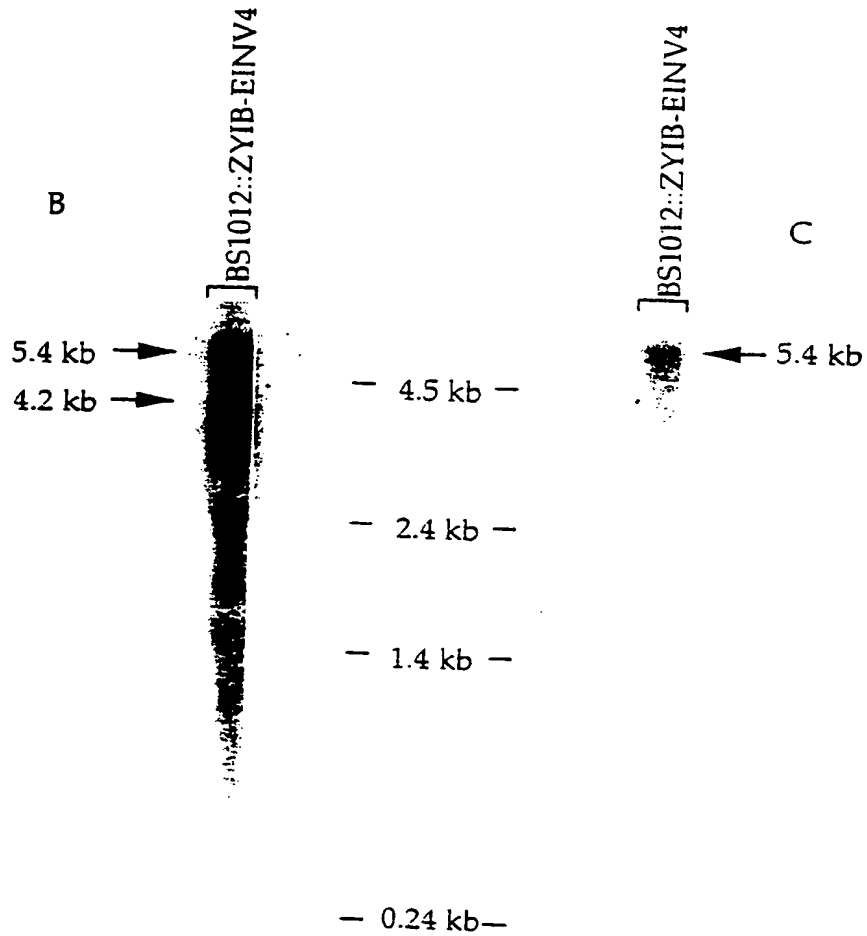


Fig. 22

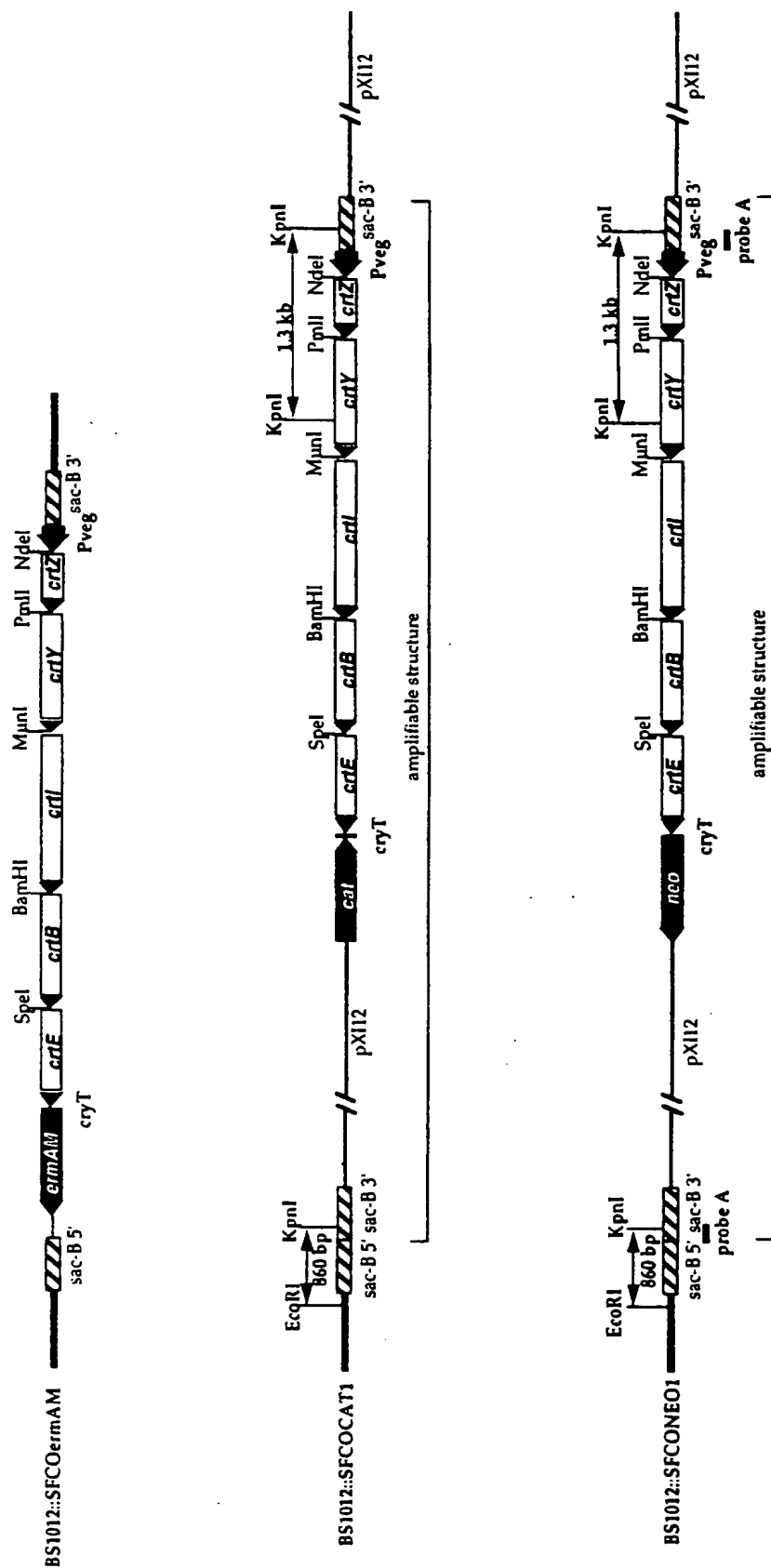


Fig. 23

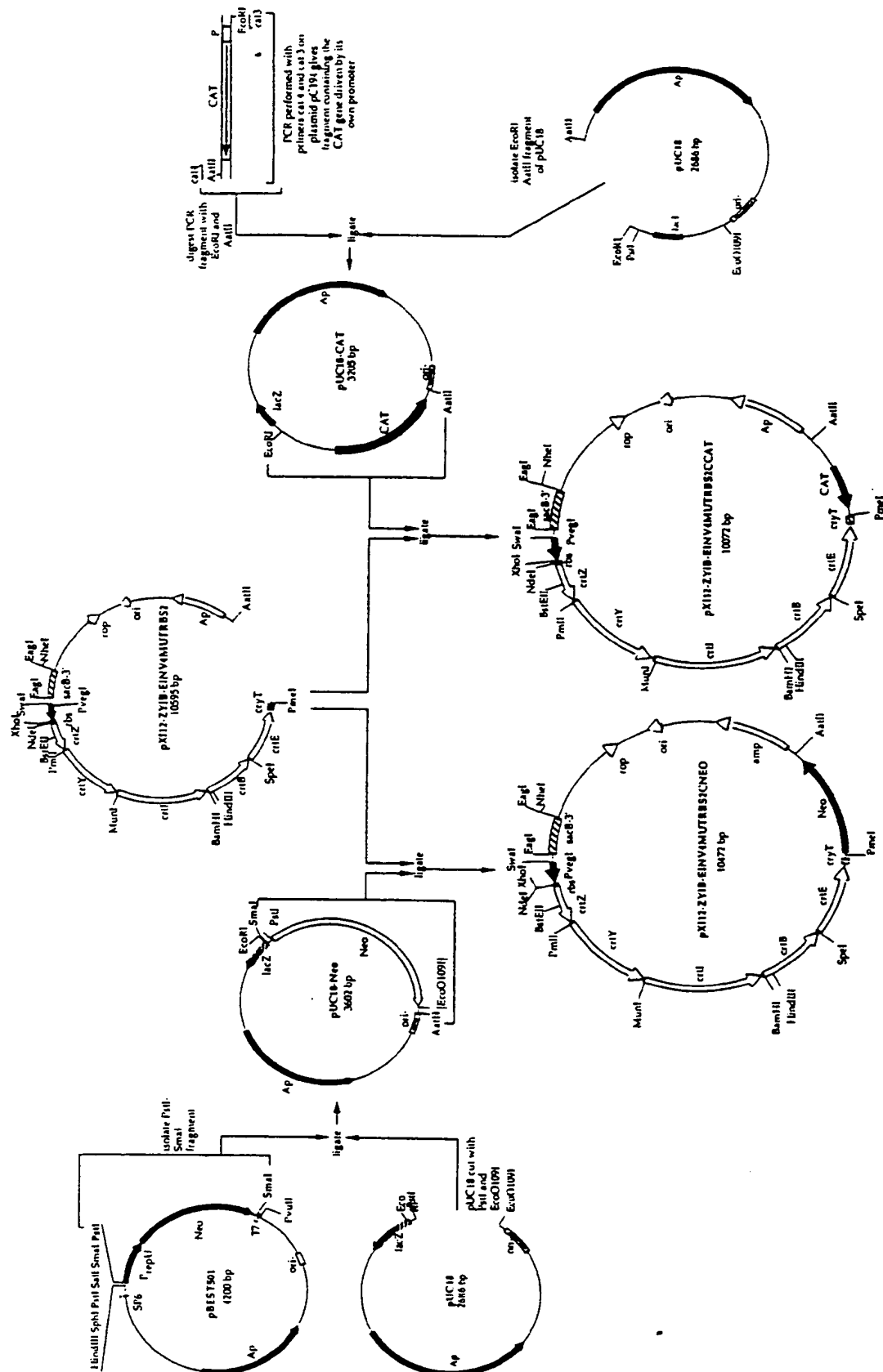


Fig. 24/1

```

1  CTAAATTGTAAGCGTTAATATTTTGTAAATTCGCGTTAAATTTTGTAAATCAGCTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
61  GATTTAATTCGCAATTATAAAACAATTTTAAAGCGCAATTTAAAAACAATTTAGTCGAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
121  ATTTTTTAACCAATAGGCCGAAATCGGCCAAAATCCCTTATAAATCAAAGAATAGACCGA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
181  TAAAAAATTGGTTATCCGGCTTAGCCGTTTAGGGAATATTTAGTTTCTTATCTGGCT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
241  GATAGGGTTGAGTGTGTTCCAGTTTGAACAAGAGTCCACTATTAAAGAACGTGGACTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
301  CTATCCCAACTCACAACAAGGTCAAACCTTGTCTCAGGTGATAATTCTTGACCTGAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
361  CAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
421  GTTGCAAGTTTCCCGCTTTTGGCAGATAGTCCCGCTACCGGGTGATGCACTTGGTAGTGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
481  CTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCTAAAGGGAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
541  GATTAGTTCAAAAAACCCAGCTCCACGGCATTTCGTGATTTAGCCTTGGGATTTCCCTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
601  CCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGAAGAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
661  GGGGGCTAAATCTCGAACTGCCCTTTTCGGCCGCTTGACCCGCTCTTTCCTTCCCTTCTT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
721  AGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
781  TCGCTTTCCTCGCCCGGATCCCGCGACCGTTTACATCGCCAGTGCGACGCGCATTGGTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
841  CACACCCGCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTAGGGCTGCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
901  GTGTGGGCGGCGCAATTACGCGCGGATGTCCCGCGCAGGGTAAGCGGTAAGTCCGACGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
961  CAAGTGTGGAAGGGCGATCGGTGCGGGCCTTTCGCTATTACGCCAGCTGGCGAAAGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1021  GTTGACAACCTTCCCGCTAGCCACGCCCGGAGAAGCGATAATGCGGTGACCCGCTTTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1081  GGGATGTGCTGCAAGGCGATTAAAGTTGGGTAACGCCAGGGTTTTCCAGTCACGACGTTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1141  CCTACACGACGTTCCGCTAATTCAACCCATTGCGGTCCCAAAGGGTCAGTGCTGCAAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1201  TAAACGACGGCCAGTGAGCGCGCTAATACGACTCACTATAGGGCGAATTGGAGCTCCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1261  ATTTTGCTGCCGGTCACTCGCGCGCATATGCTGAGTGATATCCCGCTTAACCTCGAGGT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1321  CCGCGGTGGCGGCCGCTCTAGTGGATCCGCGCCTGGCCGTTCCGATCAGCAGCCGCCCT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1381  GGCGCCACCGCGCGGAGATCACCTAGGCGCGGACCGCAAGCGCTAGTCGTGCGCGGGA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1441  TGGGATCGGTGAGCATCATCCCCATGAACCGCAGCGCAGACGCGAGCGCGCGCCCGAGA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1501  ACGCCTAGCCAGTCTAGTAGGGTACTTGGCGTCCGCTGCTGCGTCCGCGCGGGGTCT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1561  TCGGGCGCGTCCAGCACGGCATGCGCCATCATCGCGAAGGCCCGCGCGCATGGGGCGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1621  AGCCCCGCGCAGGTCGTGCCGTACGCGGTAGTAGCGTTCCGGGGGCGCGCGTACCCCGCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1681  GTGCCATTCCGAAGAACTCGCAGCCTGTCCGCTGCGCAAGGTGCGGCCAGATCGCGCCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1741  CACGGTAAGGCTTCTTGAGCGTCGGACAGGCGACGCGTTCCAGCGCGGTCTAGCGCGGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1801  TATTCGATGCACTGACGGGGCCGATGCGCGTGGGGCCGCCCTGCCCGCGGCCACCAGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1861  ATAAGGCTACGTCACTGCCCGGGCTACGCGCACCCGGCGGGACGGGGCGCGGTGGTCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

```

66

67

68

Fig. 24/5

4021 GTTCCGCGGGGGCAGATCGCGGACCTGCTGSCCCGCGTGCTGCCGCATGACATCCGCGG
 CAAGGCGCCCCCGTCTAGCGCCTGGACGACCGGGCGCACGACGGCGTACTGTAGGCGGC 4080
 4081 CAGCGCCTAGGCGCGCGGTCCGGTCCACAGGCCGTCCGCGGTGATTTGCCCGCGCGCGCAG
 GTCGCGGATCCGCGCGCCAGCCAGGTGTCCGGCAGCGCCGACTAAAGCGGCGGCGCGTC 4140
 4141 GCGCGATGCGGCCGCGTCCAAGCCTCCGCGCGCCAGAAGCCCGATCTTGGCAGCCTTCGA
 CGCGCTACGCCGCGCAGGTTCGGAGGCGCGCGGTCTTCGGGCTAGAACCCTCGGAAGCT 4200
 4201 CGTGCTGATCCGCTGGCGATAGGCCTCGGGGCCACCTGCCGGATGCGCGTCCCGATTGC
 GCACGACTAGCGGACCGCTATCCGGAGCCCCGGTGGGACGGCCTACGCGCAGGGCTAACG 4260
 4261 GCGATAGATACGACGCGCGCGCGGATCGACCACGCGCAGCGCGGCGGCGAGATGCGGAAG
 CGCTATCTATGCGTCGCGCGCGCGCTAGCTGGTGCGCGTCGCGCGCGCGCTCTACGCCTTC 4320
 4321 CCCCTGCCGCGCGGAGGCATAATAGGGCTCGGCGCGCTCAAGCAGCGCGATGATGACGGA
 GGGGACGGCGCGGCTCCGTATTATCCCGAGCGCGCGCAGTTGCTCCGCTACTACTGCCT 4380
 4381 ATAGAGCGCGTCCGAAGGCACCGGACCCCTCAACCGTCGCCCCCGCCTCGGCCAGCCAGTC
 TATCTCGCGCAGGCTTCCGTGGCCTGGGAGTTGGCAGCGGGGCGGAGCCGGTCGGTCAG 4440
 4441 GGCAGGCAGATAGCAGCGCCCGATGGCGGCATCGTCGATCACGTGCGGAGCGATGTTTCGT
 CCGTCCGTCTATCGTCGCGGGCTACCGCCGTAGCAGCTAGTGACGCGCTCGCTACAAGCA 4500
 4501 CAGCTGGAACGCAAGGCCCAGATCGCAGGCGCGATCCAGCACCGCATCGTCTTGACGCGC
 GTCGACCTTGCGTTCCGGGTCTAGCGTCCGCGCTAGGTGCTGGCGTAGCAGGACGTGCGG 4560
 4561 CATCACCCGCGCCATCATCACGCCCCAGACCCCGCGACGTGGTAGGAATATTCAGCAC
 GTAGTGGGCGCGGTAGTAGTGCGGGTGCTGGGGGCGCTGCACCATCCTTATAAGGTCGTG 4620
 4621 GTCATCCAGGCTGCGGTATTGCGGATCCGCGACATCCATCGCGAAACCCCTCGATCAGGTC
 CAGTAGGTCCGACGCCATAAGCGCTAGGCGCTGTAGGTAGCGCTTTGGGAGCTAGTCCAG 4680
 4681 CATCGGCCAAAGGTCCGGGAAATCATGCCGCGGGCGACCTGGCGCAGCGCGCGGAAGGG
 GTAGCCGGTTTCCAGGCCCTTAGTACGGCGGCGCGCTGGACCGGTGCGGCGGCTTCCC 4740
 4741 CGGCGACATCGGGCCGTCTCGTGACGCGCGCCAGCGTGTGCGCGCGCAGCGCCCCAG
 GCCGCTGTAGCCCGGCGAGGACGTCGCGCGCGGTGCGACAGCCGCGCTCGCGGGGGTC 4800
 4801 CCGCGCCTGTGGGTGCGCGCCCGCCTCGGGGCGAGAAGCCATCACCTGCCCGTCGATCAC
 GGCGCGGACACCCAGCGCGGGCGGAGCCCCGTCTTGGGTAGTGGACGGGCGAGTAGTG 4860
 4861 GTCATCCGCGATGCTTGACCCAGGCATAGAGCATGACCGTATCCTCGCGGATGCCGGGCGG
 CAGTAGGCGTACGGACGTGCTCGTATCTCGTACTGGCATAGGAGCGCCTACGGCCCCGC 4920
 4921 CATCAGCTTGGCCGCTGCGCGAAGCTTTGCGAACCCTGCGCGATGGCCGCTTCGGAAGT
 GTAGTCGAACCGGCGGACGCGCTTCGAAACGCTTGGGACGCGCTACCGGCGAAGCCTTCA 4980
 4981 CGCCGTGAGATCGGTGATGCGACGGCCAGGTCCGACAGCATGACCTGCGCCGTGGCCCTTG
 GCGGCAGTCTAGCCAGTACGCTGCCGGTCCAGGCTGTCGTAAGTGGACGCGGACCGGAAC 5040

Fig. 24/6

5041 GCGCTGCCAACGACACCCGGGATGCCCGCACCCGGATGCGTGCCCGCCCCACGATGTAG 5100
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 CGCGACGGTTGCTGTGGGCCCTACGGGCGTGGGCCCTACGCACGGGCGGGGGTGTACATC
 5101 AAGTTCGGGATCGCGCGGTTCGCGTTATGCGGGCGGAACCAGGCGGATTGCGTCAGGATC 5160
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TTCAAGCCCTAGCGCGCCAGCGCCAATACGCCCGCCTTGGTCCGCCTAACGCAGTCCTAG
 5161 GGCTCGACCGAGAAGGCGCTGCCGTGATGGGCCGACAGTTCGGTGCTGAAATCGGCGGGG 5220
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 CCGAGCTGGCTCTTCGCGACGGCACTACCGGGCTGTCAAGCCACGACTTTAGCCGCCCC
 5221 CTGAAGATGCGGCTGACGGTCAGGTGCTTGCAGGTTCGGGGATGGCGCGGCGCTCCAGT 5280
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 GACTTCTACGCCGACTGCCAGTCCACGAACGCGTCCAGCCCCCTACCGCGCCGCGAGGTCA
 5281 TCCTCGAAGATGCGCTCGGCATAGCCCGGGGCCCTCGGCTTCCCAATCGACATCGGCGCGG 5340
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 AGGAGCTTCTACGCGAGCCGTATCGGGCCCCGGAGCCGAAGGGTTAGCTGTAGCCGCGCC
 5341 CCCAGATGCGGAACGGGCGCAAGGACGTAATGCGTGGACATCCCCCTCGGGGGCCAGGCTG 5400
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 GGGTCTACGCCTTGCCCGCGTTCTCTGCATTACGCACCTGTAGGGGAGCCCCCGGTCCGAC
 5401 GGATCGGTACGCAGGGCGAATGCAGATACATCGAGAAATCGTCCGGCAGGCGTGGCCCCG 5460
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 CCTAGCCAGTGGTCCCGCTTACGTCTATGTAGCTCTTTAGCAGGCGGTCCGCACCGGGC
 5461 TTGAAGATCTCGTTCACCAGCCCCCTTGTAGCGGGGCCGAAGATGACGCTGTGGTGGGCC 5520
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 AACTTCTAGAGCAAGTGGTCGGGAACATCGCGCCCCGGCTTCTACTCGACACCACCCGG
 5521 AGGTTCTCGGGGCGCTTGGACAGGCCGAAATGCAGCACGAACAGCGACATCGACCAGCGC 5580
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TCCAAGAGCCCCGGAACCTGTCCGGCTTACGTCTGCTGCTTGTGCTGTAGCTGGTCCGC
 5581 TGCCGGTTCAGGATCGCGGCCTTGGTGGCCCCGCGCGGGTATGGCCCAGCAGGTCCGGA 5640
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 ACGGCCAAGTCTAGCGCCGGAACACGCGGGCGCCGCCCATACCGGGTCGTCCAGCGCT
 5641 TAGCTGTGCATCAGTTCGCCGTGCTGGCCACCGTATCCGCGCGCAACTGCCGCCCGTCC 5700
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 ATCGACACGTAGTGCAGCGGCAACGACCGGTGGCATAGGCGCGCGTTGACGGCGGGCAGG
 5701 AGCAGCGTGACGCCCCGTGGCGCGATCGCCCTCGGTGTGATCCGCGTGACGCGGGCATTC 5760
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TCGTTCGCACTGCGGGCACCGGCTAGCGGGAGCCACAGCTAGGCGCACTGCGCCCCGTAAG
 5761 AGCAGCAGCGTGCCGCCAAGACGCTCGAACAGGGCGACCATGCCCGCGACCAGCTGGTTG 5820
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TCGTTCGTCGACGGCGGTCTGCGAGCTTGTCCCGCTGGTACGGGCGCTGGTTCGACCAAC
 5821 GTGCCGCCCTTGGCGAACAGACGCGCGCCGCGCGCTTCCAGCGCATGGATCAGCGCATAG 5880
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 CACGGCGGGAACCGCTTGGTCTGCGGCGGCGCGGCAAGGTCCGCTACCTAGTCGCGTATC
 5881 ATCGAGCTGGTTCGAAAACGGGTTCGCCCGGACAGCAGCGTGTGGAACGAGAAGGCCTGC 5940
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TAGCTCGACCAAGCTTTGCCCAAGGGCGGCTGGTTCGTCGACACCTTGCTCTTCCGGACG
 5941 CGCAGATGCGGGTCTTGATGAAGCGCGCCACCATGCTGTGGACCGAGCGGTATGCCTGC 6000
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 GCGTCTACGCCCAGGACCTACTTCGCGCGGTGGTACGACACCTGGCTCGCCATACGGACG
 6001 AGGCGCATCAGCGCCGCGCGGCGGCTTCAGCATCTGCCCGAGCTTCAGGAAGGGCGTGGTC 6060
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TCCGCGTAGTCGCGGCGCGCGCGGCAAGTCTGTAGACCGGGTCAAGTCCTTCCCGCACCAAG

Fig. 24/7

6061 CCCAGCTTCAGATACCCCTCGCGATAGACCTCCTCGGCGTAATCGTGGAGCGGCGATAG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6120
 GGGTCGAAGTCTATGGGGAGCGCTATCTGGAGGAGCCGATTAGCACCTTCGCGCGCTATC
 6121 CCATCGACATCGGCGGGATTGAAGGAGGCGACCTGGCGGATCAGCTCGTCGTCGTCGTTT
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6180
 GGTAGCTGTAGCCGCCCTAACTTCCTCCGCTGGACCGGCTAGTCGAGCAGCAGCAGCAAG
 6181 ACGTATTCTGAAGCTGCGGCCGTCCGCCCATGTCAGCCGGTAGAAGGGCGAGACCGGCGAGC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6240
 TGCATAAGCTTCGACGCGCGGCGGCGGTACAGTCGCCCATCTTCCCGCTCTGGCCGTCTG
 6241 AGCGTCACGTCACGCTCCATCGGTTGGCCGCTGAGGGCCACAGCTCTCGCAGGCTGTCTG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6300
 TCGCAGTGCACTGCGAGGTAGCCAACCGGCGACTCCCGGGTGTGAGAGCGTCCGACAGC
 6301 GGGTCGGTCACGACCGTCGGGCTGTCATCGAAGACGTGGCCCTGATCGTTCCAGACATAG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6360
 CCCAGCCAGTGCTGGCAGCCCGGACGTAGCTTCTGCACCGGGACTAGCAAGGTCTGTATC
 6361 GCGCGGCCCGCGGGCTTGTGCGGGCCCTCGACGATGGTGGTCGCGATGCCGGCCGATTGC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6420
 CGCGCCGGCGGCGCCGAACAGCGCCCGGAGCTGCTACCAACAGCGCTACGGCCGGCTAACG
 6421 AGGCGGATGGCAAGCGCAAGCCCGCGGAACTGCGCCGATGACGATGGCGGAACTCATG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6480
 TCCGCCTACCGTTTCGCGTTCGGGCGGCTTTGGACGCGGCTACTGCTACCGCCTTGAGTAC
 6481 CTCTCTCCTGCAGCAGGGGGCGTTCGGGCAGGCGCGCACGGCCTGCGACAGCGGAATGG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6540
 GAGAGAGGACGTGCTCCCCCGCAAGCCCGTCCGTGCGGTGCCGGACGCTGTGCGCTTACC
 6541 GCGGGCGTCCGGTGACGATGCGAAGCCGGTCCGCCAATGTCAGGCGCCCGGCATAGAAGC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6600
 CGCCCGCAGGCCACTGCTACGCTTCGGCCAGCCGGTTACAGTCCGCGGGCCGTATCTTCG
 6601 GCTCGATCAGCGGCTGCGGCAGGCGGTAGAACCGCTGCAGCAGGCGATAGCGACGGTCCG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6660
 CGAGCTAGTCGCGGACGCCGTCCGCCATCTTGCGGACGTGCTCCGCTATCGCTGCCAGCC
 6661 GCGGGCAGCCCGGGAACAGCATCCGGTTGAGCAGCCGCGAGGAAGCGGTGCGGATCCGCGC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6720
 CGCCCGTCCGGCGCCTTGTGCTAGGCCAAGTCGTCGGCGTCTTCGCCAGCGCTAGGCGCG
 6721 GATCGATGGCCAGCCCGCACCGCGCGACGGGCGGACGCGGTGCTCAGGTGCGCGCGCG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6780
 CTAGCTACCGGGTCCGGCGGTGGCGCGCTGCCCGCCTGCGCCAGCAGTCCAGCGCGCGCG
 6781 CGATGGCATCCGCGACCTGCGCGGCATAGGGCAGCGAATATCCGGTGACGGGGTGGAAAC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6840
 GCTACCGTAGGCGCTGGACGCGCGGTATCCCGTTCGCTTATAGGCCACTGCCCGACCTTGT
 6841 GCCCTGCCCCCAGCCCAACCGGCACCGCCCCCTGCGCGTGGTCCGCCAGAAGCCTATGG
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6900
 CGGGACGGGGGTGGGTTGGCGGTGGCGGGGACGCGCACCGCGCGGTCTTCGGATACC
 6901 CGTCATGGGCCAGCGCGATGGGCAGGATGCCCCCTTCGCGCCGATCTCTGCCCGGTCC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 6960
 GCAGTACCGGGTCCGCTACCGCTCTACGGGAAAGCGCGCGGTAGAGGACGGGCCAGG
 6961 AGCCCCCGCTGGCGGCATAGTCCAGCGACGCGTGGCCAGCGCGCCATCGTCCAGATCGC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 7020
 TCGGGCGGACCGCGGTATCAGGTGCTGCGGACGCGGTGCGCGGTAGCAGGTCTAGCG

Fig. 24/8

```

CGCCGTCGCTGTAGCGCGTATCCTCGATCAGGATGCGGGTGGGACTGAAGGGCAGCAGAT
7021 -----+-----+-----+-----+-----+-----+-----+-----+ 7080
GCGGCAGCGACATCGCGCATAGGAGCTAGTCCTACGCCACCCTGACTTCCCGTCGTCTA

AGATGAAGCGGTACCCGTCCATCTGCGGAACGGTCGCGTCCATGATCATCGGGCGCTCGA
7081 -----+-----+-----+-----+-----+-----+-----+-----+ 7140
TCTACTTCGCCATGGGCAGGTAGACGCCTTGCCAGCGCAGGTACTAGTAGCCCGCGAGCT

CGCCATGGGGGGCGTCGGTCTCGATCTCGACGCCCCACGAATTTCTGGAAACCCACGGTCA
7141 -----+-----+-----+-----+-----+-----+-----+-----+ 7200
GCGGTACCCCCCGCAGCCAGAGCTAGAGCTGCGGGTGCTTAAAGACCTTTGGGTGCCAGT

GGTGGGGGTCTCGACGGCACCACGGGCGTCGATCACGCAGGCAGCCTCGATCCGCGAGC
7201 -----+-----+-----+-----+-----+-----+-----+-----+ 7260
CCACGCCCCAGAGCTGCCGTGGTSCCCGAGCTAGTGCGTCCGTGGGAGCTAGGCGCTCG

CGTCCGTCAGCGTCGCGCCGGTATCGTCCAGCGTCGCGACATGCGTATTCCACCGCAGAT
7261 -----+-----+-----+-----+-----+-----+-----+-----+ 7320
GCAGGCAGTCGCAGCGCGGCCATAGCAGGTGCGAGCGCTGTACGCATAAGGTGGCGTCTA

CGACACCCTGCAGCAGCCCGATCAGCGCGCCCGCCTCGATCGAGCCATAGCCTGTCTGTC
7321 -----+-----+-----+-----+-----+-----+-----+-----+ 7380
GCTGTGGGACGTCGTCGGGCTAGTCGCGCGGGCGGAGCTAGCTCCGTATCGGACAGCAGT

GGCGGCGCGAATGGTCGGGAACCGGACCTCCTGATCCGTCCATTCCGCGCGACGAATGG
7381 -----+-----+-----+-----+-----+-----+-----+-----+ 7440
CCGCGCGGCTTACCAGCCCTTTCGCTGGAGGACTAGGCAGGTAAGCGGCGCTGCTTACC

GCGACAGGCGCGCCAGCCATTGGGCGAAAGATCCGTGTCTGGCAGGACCAGGTGTGCT
7441 -----+-----+-----+-----+-----+-----+-----+-----+ 7500
CGCTGTCCGCGCGGTGCGTAAGCCCGCTTTCTAGGCACAGCACCGTCCTGGTCCACACGA

GGTCCGAGGGGCGCGACCGCGCGTCGAGCATCAGATGCGCGCATCCGGTCTGCGGTGCG
7501 -----+-----+-----+-----+-----+-----+-----+-----+ 7560
CCAGGCTCCCCGGCCTGGCGCGCAGCTCGTAGTGCTACGCGCGTAGGCCAGACGCCAGCG

GAACGGCAAGCGCGATCAGCGCACCGGACAGCCCCGCGCCCGGATCAGCAGATCATGGC
7561 -----+-----+-----+-----+-----+-----+-----+-----+ 7620
CTTGCCSTTCGCGCTAGTCGCGTGGCCTGTGGGGCGCGGGCGCTAGTCGTCTAGTACCG

TCATGTATTGCGATCCGCCCTTCGCGGTCTTCAGCAGCGCGCCCGAGCGTTTCAGCTC
7621 -----+-----+-----+-----+-----+-----+-----+-----+ 7680
AGTACATAACGCTAGGCGGGGAAGCGCCAGGAAGTCGTGCGCGGGGCTCGCAAAGTCGAG

TGCCTTGAGGCTGTGACCGAGGGCGCCAGATGAAACCGAAGCTGACGCAGTTCTCGCG
7681 -----+-----+-----+-----+-----+-----+-----+-----+ 7740
ACGGAACCTCCGACAGCTGGCTCCCGCGGGTCTACTTTGGCTTCGACTGCGTCAAGAGCGC

GCCATGGACCGCGTGATGCATCCTGTGTGCTGGTAGACGCGACGAAGATAGCCGCGCTT
7741 -----+-----+-----+-----+-----+-----+-----+-----+ 7800
CGGTACCTGGCGCACTACGTAGGACACACGGACCATCTGCGCTGCTTCTATCGGCGCGAA

GGGGACATAGCGGAACGGCCAGCGCCCATGCACCAAGCCGTCATGCAGGAAATAGTAGAT
7801 -----+-----+-----+-----+-----+-----+-----+-----+ 7860
CCCCTGTATCGCCTTGCCGGTCGCGGTACGTGGTTCGGCAGTACGTCTTTATCATCTA

CAGCCCGTAGCAGGTGACCCCCACCGCCAGCCACCAGGCCAGATCCGACCCCATCGCGCC
7861 -----+-----+-----+-----+-----+-----+-----+-----+ 7920
GTGCGGCATCGTCCACTGGGGGTGGCGGTGCGTGGTCCGGTCTAGGCTGGGGTAGCGCGG

GATCGCGAACAGCACGATCGAGATTACCGCGAAGATGACGCCATAGAGGTCGTTCTTCTC
7921 -----+-----+-----+-----+-----+-----+-----+-----+ 7980
CTAGCGCTTGTGCTGCTAGCTCTAATGGCGCTTCTACTGCGGTATCTCCAGCAAGAAGAG

```


Fig. 24/10

```

9001 ATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTGTTCCCTTTAGTGAGGGTTA
-----+-----+-----+-----+-----+-----+-----+-----+
9060 TATGGCAGCTGGAGCTCCCCCGGGCCATGGGTCGAAAACAAGGGAAATCACTCCCAAT
-----+-----+-----+-----+-----+-----+-----+-----+

9061 ATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTC
-----+-----+-----+-----+-----+-----+-----+-----+
9120 TAACGCGGAACCGCATTAGTACCAGTATCGACAAAGGACACACTTTAACAATAGGCGAG
-----+-----+-----+-----+-----+-----+-----+-----+

9121 ACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGA
-----+-----+-----+-----+-----+-----+-----+-----+
9180 TGTTAAGGTGTGTTGTATGCTCGGCCTTCGTATTTTCACATTTGAGACCCACGGATTACT
-----+-----+-----+-----+-----+-----+-----+-----+

9181 GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCCGCTTCCAGTCGGGAAACCTG
-----+-----+-----+-----+-----+-----+-----+-----+
9240 CACTCGATTGAGTGAATTAACGCAACGCGAGTGACGGGCGAAAGGTCAGCCCTTTGGAC
-----+-----+-----+-----+-----+-----+-----+-----+

9241 TCGTGCCAGCTGCATTAAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATTGGG
-----+-----+-----+-----+-----+-----+-----+-----+
9300 AGCACGGTCGACGTAATTACTTAGCCGGTTGCGCGCCCTCTCCGCCAAACGCATAACCC
-----+-----+-----+-----+-----+-----+-----+-----+

9301 CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTTCGGCTGCGGCGAGCG
-----+-----+-----+-----+-----+-----+-----+-----+
9360 GCGAGAAGGCCAAGGAGCGAGTGACTGAGCGACGCGAGCCAGCAAGCCGACGCGCTCGC
-----+-----+-----+-----+-----+-----+-----+-----+

9361 GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAAACAGGGGATAACGCAGGA
-----+-----+-----+-----+-----+-----+-----+-----+
9420 CATAGTCGAGTGAGTTTCGCCATTATGCCAATAGGTGTCTTAGTCCCCTATTGCGTCTT
-----+-----+-----+-----+-----+-----+-----+-----+

9421 AAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTG
-----+-----+-----+-----+-----+-----+-----+-----+
9480 TTCTTGTACTCGTTTTCCGGTCGTTTTCCGGTCCTTGGCATTTTCCGGCGCAACGAC
-----+-----+-----+-----+-----+-----+-----+-----+

9481 GCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG
-----+-----+-----+-----+-----+-----+-----+-----+
9540 CGCAAAAAGGTATCCGAGGCGGGGGGACTGCTCGTAGTGTTTTAGCTGCGAGTTCAGTC
-----+-----+-----+-----+-----+-----+-----+-----+

9541 AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC
-----+-----+-----+-----+-----+-----+-----+-----+
9600 TCCACCGCTTTGGGCTGTCTGATATTCTATGGTCCGCAAGGGGGACCTTCGAGGGAG
-----+-----+-----+-----+-----+-----+-----+-----+

9601 GTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCG
-----+-----+-----+-----+-----+-----+-----+-----+
9660 CACGCGAGAGGACAAGGCTGGGACGGCGAATGGCCTATGGACAGGCGGAAAGAGGGAAGC
-----+-----+-----+-----+-----+-----+-----+-----+

9661 GGAAGCGTGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTGTT
-----+-----+-----+-----+-----+-----+-----+-----+
9720 CCTTCGCACCCGCAAGAGTATCGAGTGCGACATCCATAGAGTCAAGCCACATCCAGCAA
-----+-----+-----+-----+-----+-----+-----+-----+

9721 CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTACGCCGACCGCTGCGCCTTATCC
-----+-----+-----+-----+-----+-----+-----+-----+
9780 GCGAGGTTTCGACCCGACACAGTGCTTGGGGGCAAGTCGGGCTGGCGACGCGGAATAGG
-----+-----+-----+-----+-----+-----+-----+-----+

9781 GGTAACTATCGTCTTGAGTCCAACCCGTAAGACACGACTTATCGCCACTGGCAGCAGCC
-----+-----+-----+-----+-----+-----+-----+-----+
9840 CCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGACCGTCGTCCG
-----+-----+-----+-----+-----+-----+-----+-----+

9841 ACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGG
-----+-----+-----+-----+-----+-----+-----+-----+
9900 TGACCATTGCTCTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCACC
-----+-----+-----+-----+-----+-----+-----+-----+

9901 TGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCA
-----+-----+-----+-----+-----+-----+-----+-----+
9960 ACCGGATTGATGCCGATGTGATCTTCCTGTCTATAAACCATAGACGCGAGACGACTTCGGT
-----+-----+-----+-----+-----+-----+-----+-----+

```

Fig. 24/11

```

9961 GTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGC 10020
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
CAATGGAAGCCTTTTTCTCAACCATCGAGAAGTAGGCCGTTTGTGGTGCGGACCATCG

10021 GGTGGTTTTTTTTGTTTGAAGCAGCAGATTACGGCAGAAAAAAGGATCTCAAGAAGAT 10080
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
CCACCAAAAAAACAACGTTTCGTCTCTAATGCGCGTCTTTTTTCTAGAGTTCTTCTA

10081 CCTTTGATCTTTTCTACGGCGTCTGACGCTCAGTGAACGAAAACTCACGTTAAGGGATT 10140
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
GGAACTAGAAAAGATGCCCCAGACTGCGAGTCACCTTGCTTTTGAGTGCAATTCCTAA

10141 TTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGT 10200
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
AACCAGTACTCTAATAGTTTTTCTAGAAGTGGATCTAGGAAAAATTAATTTTACTTCA

10201 TTTAAATCAATCTAAAGTATATATAGTAAACTTGGTCTGACAGTTACCAATGCTTAATC 10260
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
AAATTTAGTTAGATTTCATATATACTCAATTTGAACCAGACTGTCAATGGTTACGAATTAG

AGTGAGGCACCTATCTCAGCGATCTGTCTATTTCTGTTTCATCCATAGTTGCCTGACTCCCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10261 TCACTCCGTGGATAGAGTCGCTAGACAGATAAAGCAAGTAGGTATCAACGGACTGAGGGG 10320
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

GTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10321 CAGCACATCTATTGATGCTATGCCCTCCCGAATGGTAGACCGGGGTACGACGTTACTAT 10380
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

CCGCGAGACCCACGCTCACGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10381 GCGGCTCTGGGTGCGAGTGGCCGAGGTCTAAATAGTCGTTATTTGGTCGGTCGGCCTTCC 10440
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

GCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAAATGTTGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10441 CGGCTCGCGTCTTACCAGGACGTTGAAATAGCGGAGGTAGGTGAGATAATTAACAACG 10500
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGGCACAACGTTGTTGCCATTGCT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10501 GCCCTTCGATCTCATTCAACAACGGTCAATATCAAACGGGTTGCAACAACGGTAACGA 10560
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

ACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTAGCTCCGGTTCCCAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10561 TGTCCGTAGCACCACAGTCCGAGCAGCAACCATACCGAAGTAAGTCGAGGCCAAGGGTT 10620
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

CGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10621 GCTAGTTCCGCTCAATGTACTAGGGGTACAACACGTTTTTTCGCCAATCGAGGAAGCCA 10680
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

CCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10681 GGAGGCTAGCAACAGTCTTCATTCAACCGGCGTCACAATAGTGAGTACCAATACCGTCGT 10740
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

CTGCATAATTCTCTTACTGTCTGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10741 GACGTATTAAGAGAATGACAGTACGGTAGGCATTCTACGAAAAGACACTGACCACTCATG 10800
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

TCAACCAAGTCATTCTGAGAATAGTGTATGCCGCGACCGAGTTGCTCTTGCCCGGCGTCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10801 AGTTGGTTCAGTAAGACTCTTATCACATACGCGCTGGCTCAACGAGAACGGGCGGAGT 10860
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

ATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGAAAAACGT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10861 TATGCCCTATTATGGCGCGGTGTATCGTCTTGAATTTTCACGAGTAGTAACCTTTTGCA 10920
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

TCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
10921 AGAAGCCCCCGTTTTGAGAGTTCCTAGAATGGCGACAACCTTAGGTCAAGCTACATTGGG 10980
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

```

Fig. 24/12

```

10981  ACTCGTGCACCCAACTGATCTTCAGCATCTTTACTTTCAACGCGTTTCTGGGTGAGCA
-----+-----+-----+-----+-----+-----+-----+
11040  TGAGCACGTGGGTTGACTAGAAAGTCGTAGAAAATGAAAGTGGTCGCAAAGACCCACTCGT
-----+-----+-----+-----+-----+-----+-----+
11041  AAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATA
-----+-----+-----+-----+-----+-----+-----+
11100  TTTTGTCTTCGTTTTACGGCGTTTTTTCCTTATTCCGGCTGTGCCTTTACAACTTAT
-----+-----+-----+-----+-----+-----+-----+
11101  CTCATACTCTTCCTTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGC
-----+-----+-----+-----+-----+-----+-----+
11160  GAGTATGAGAAGGAAAAAGTTATAATAACTTCGTAAATAGTCCCAATAACAGAGTACTCG
-----+-----+-----+-----+-----+-----+-----+
11161  GGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCC
-----+-----+-----+-----+-----+-----+-----+
11220  CCTATGTATAAACTTACATAAAATCTTTTTATTGTTTATCCCAAGGCGCGTGTAAAGGG
-----+-----+-----+-----+-----+-----+-----+
11221  CGAAAAGTGCCAC
-----+-----+-----+-----+-----+-----+-----+
11233  GCTTTTCACGGTG
-----+-----+-----+-----+-----+-----+-----+

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721 CGTGGT 726

Fig. 26

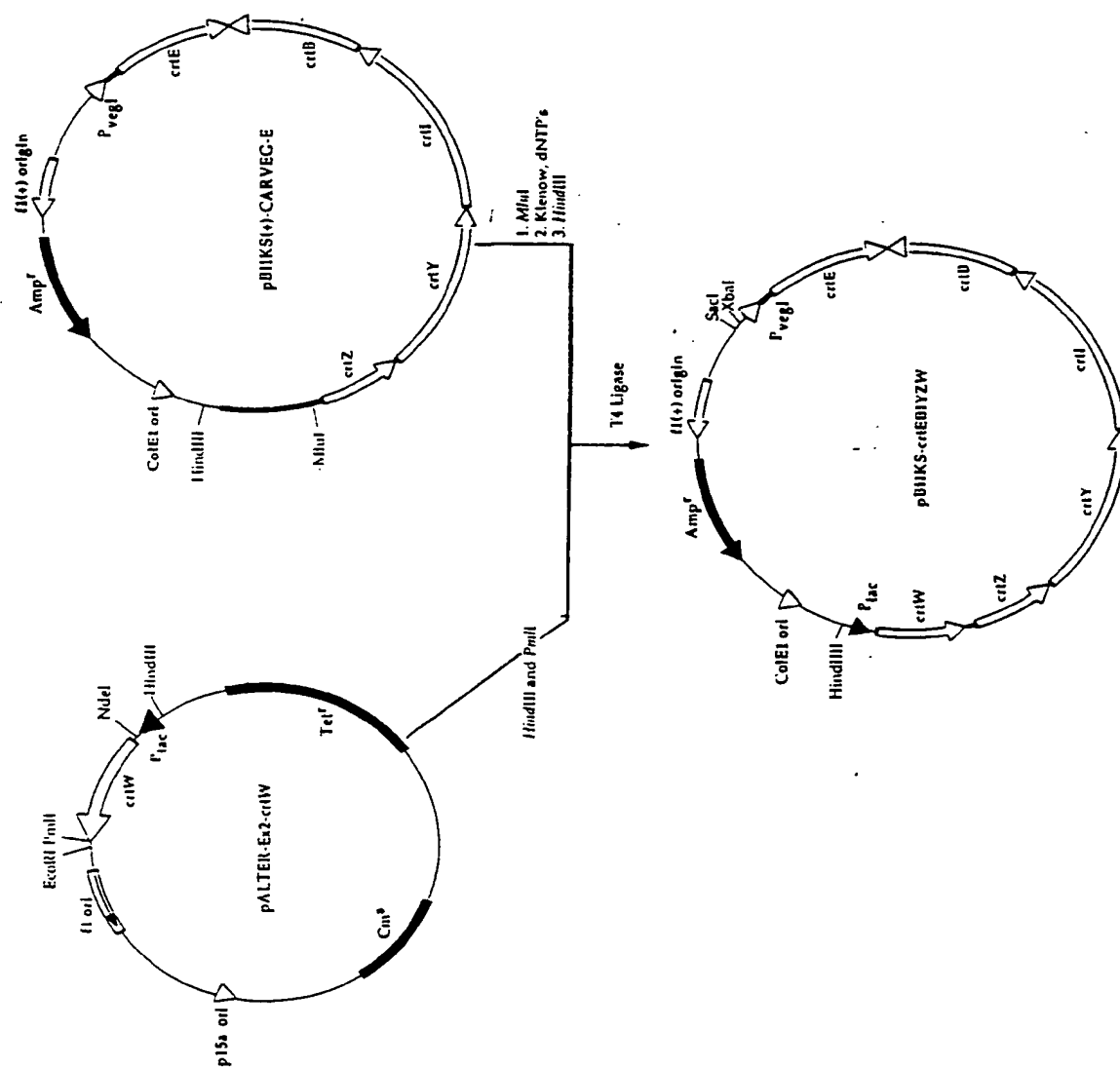


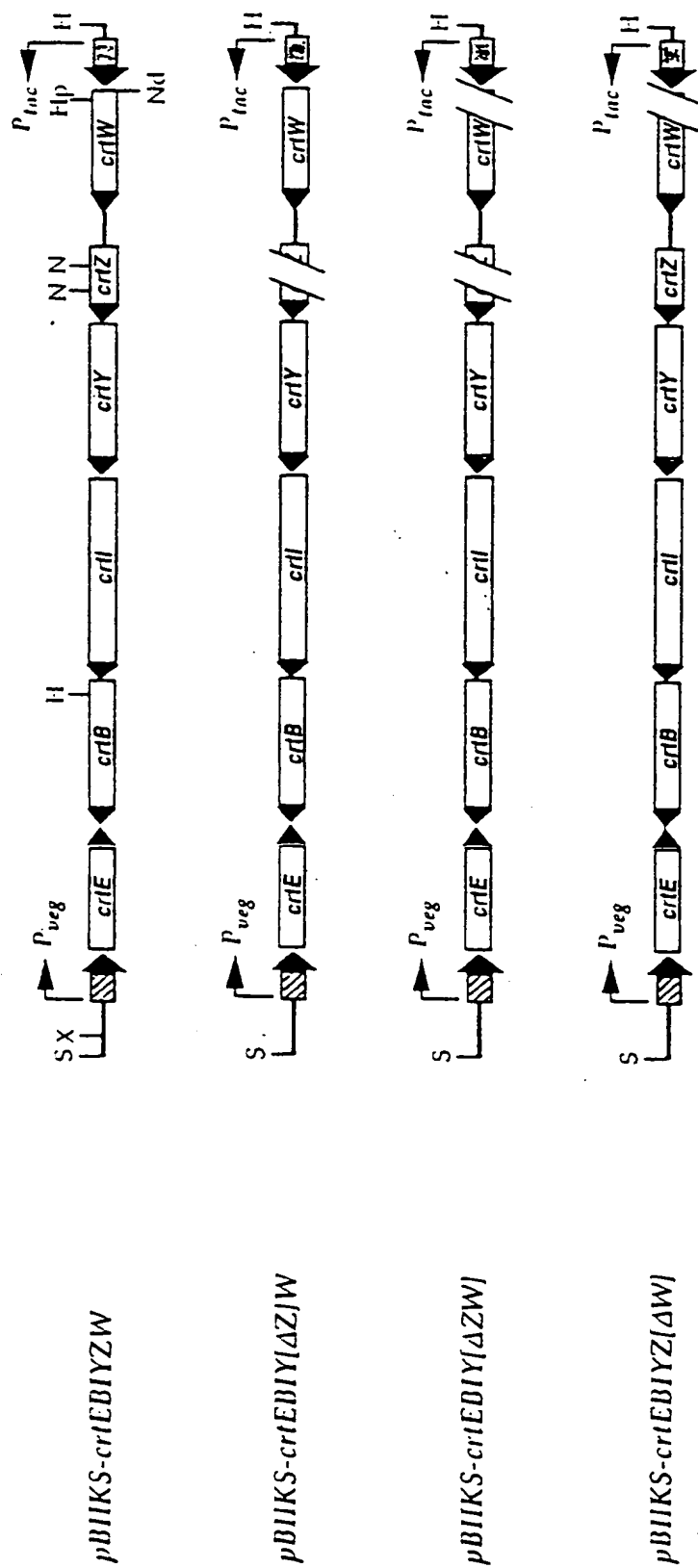
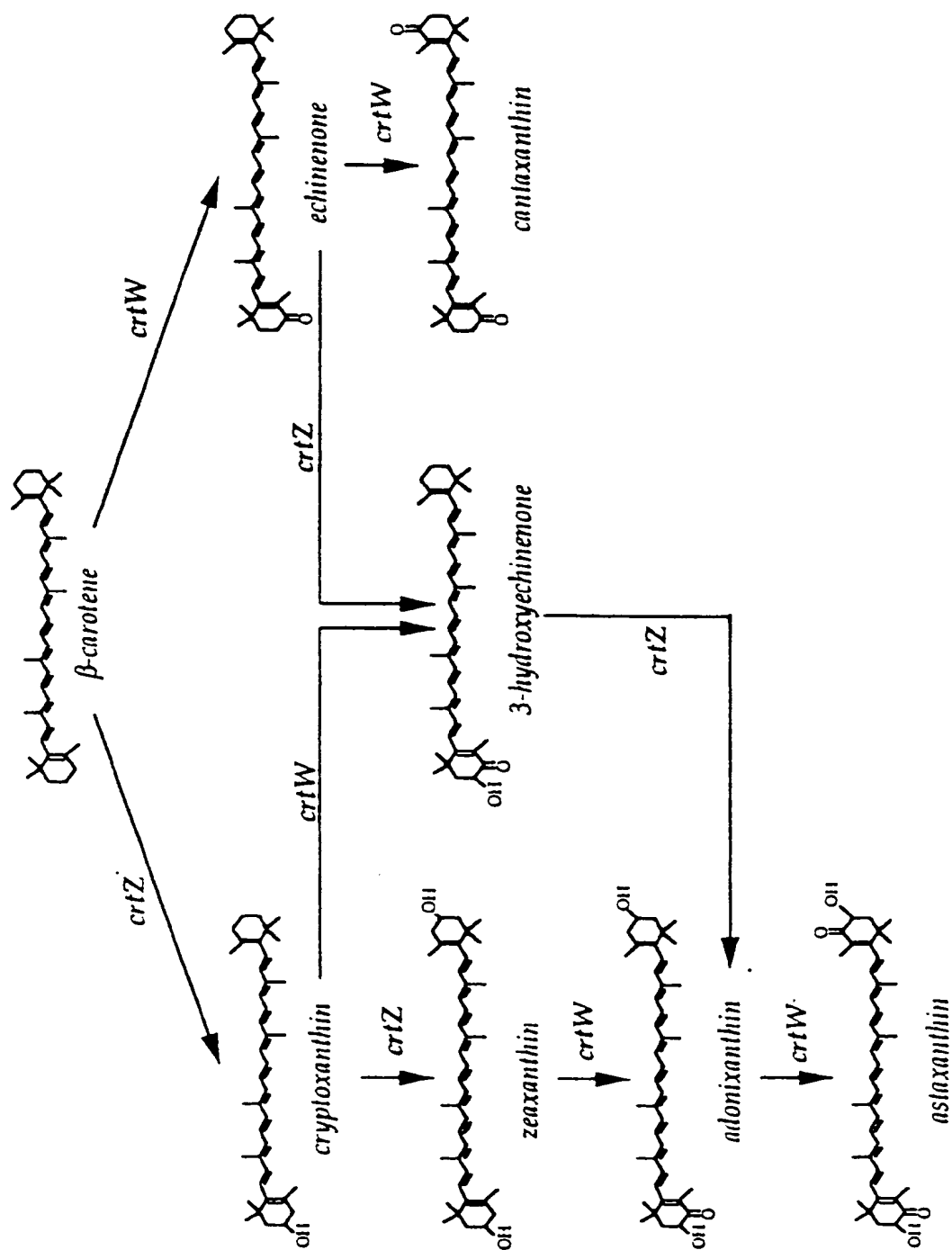
Fig. 27

Fig. 28

(19)



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(11)

EP 0 747 483 A3

(12)

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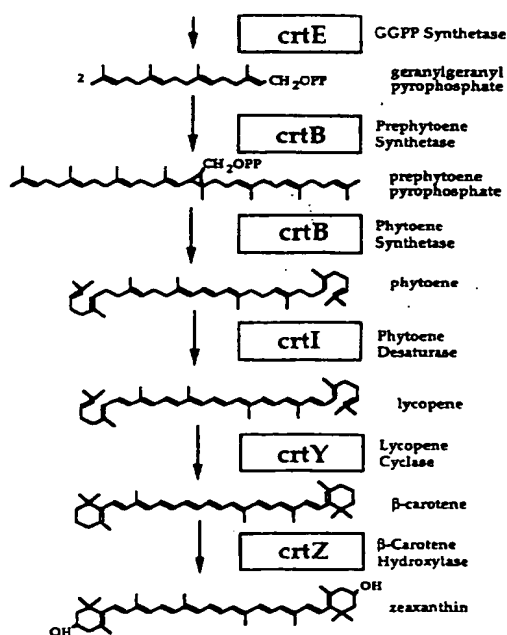
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• Tessier, Michel
68100 Mulhouse (FR)
• van Loon, Adolphus
4310 Rheinfelden (CH)

(74) Representative: Braun, Axel et al
F.Hoffmann-La Roche AG
Patent Department (PLP),
124 Grenzacherstrasse
4070 Basel (CH)

(54) Fermentative carotenoid production

(57) The present invention is directed to a DNA sequence comprising one or more DNA sequences selected from the group consisting of a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE), a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB), a DNA sequence which encodes the phytoene synthase of *Flavobacterium* sp. R1534 (crtB), a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which encodes the β -carotene hydroxylase of *Flavobacterium* sp. R1534 (crtZ) or DNA sequences which are substantially homologous, vectors comprising such DNA sequences and/or a DNA sequence which encodes the β -carotene β 4-oxygenase of *Alcaligenes* strain PC-1 (crtW) or a DNA sequence which is substantially homologous, cells which are transformed by such DNA sequences and/or vectors, a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such transformed cells and a process for the preparation of a food or feed composition.

Fig. 1



EP 0 747 483 A3



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EUROPEAN SEARCH REPORT

Application Number
EP 96 10 8556

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
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The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 6 March 1997	Examiner De Kok, A
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EUROPEAN SEARCH REPORT

Application Number
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<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>----- & : member of the same patent family, corresponding document</p>			

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